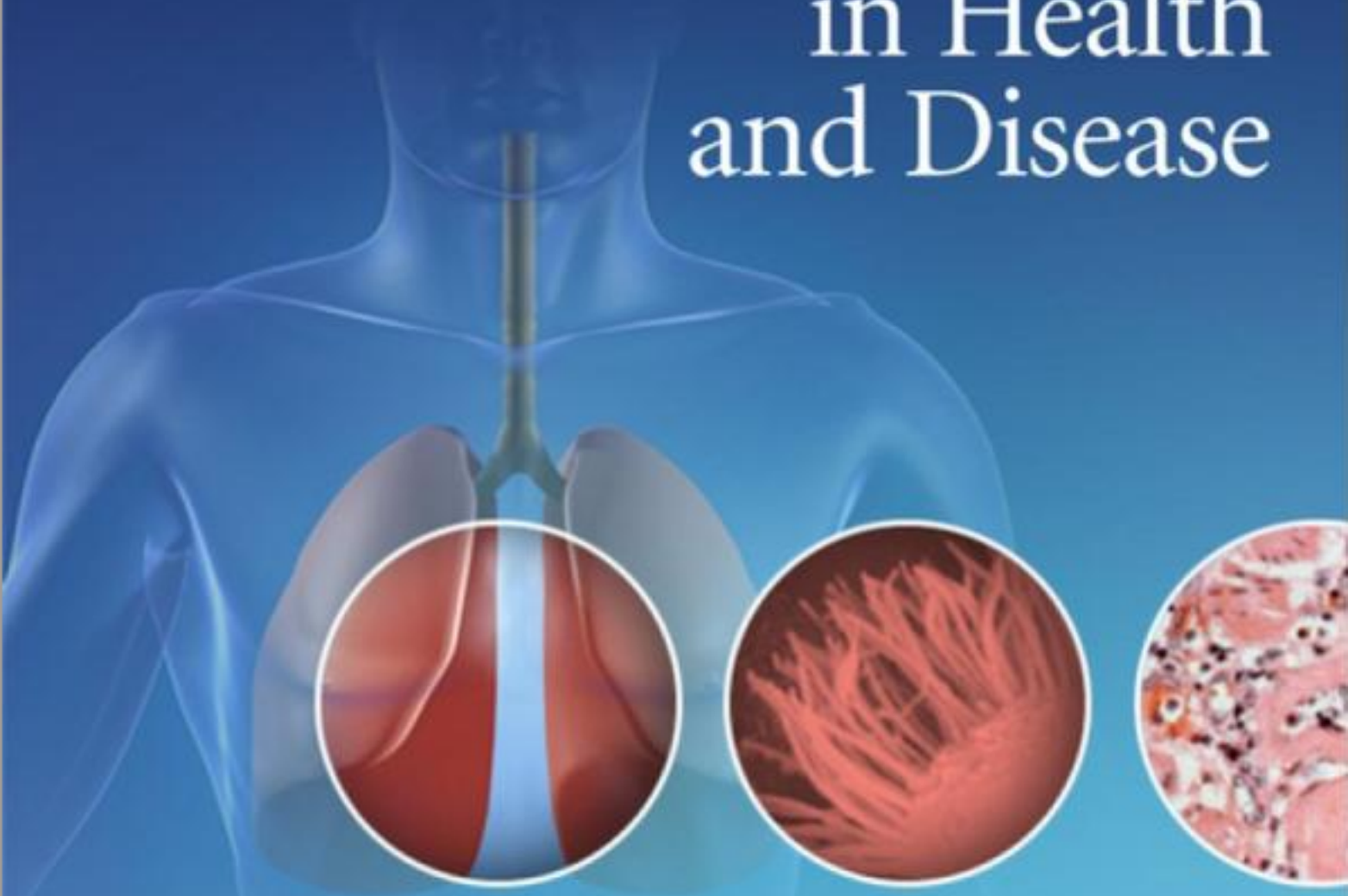
The Pulmonary Epithelium in Health and Disease



David Proud

WILEY

The Pulmonary Epithelium in Health and Disease

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

The Pulmonary Epithelium in Health and Disease

Edited by

David Proud

Department of Physiology and Biophysics,
University of Calgary, Alberta, Canada

John Wiley & Sons, Ltd

John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester,
West Sussex PO19 8SQ, England

Telephone (+44) 1243 779777

Email (for orders and customer service enquiries): cs-books@wiley.co.uk
Visit our Home Page on www.wiley.com

Other Wiley Editorial Offices

John Wiley & Sons Inc., 111 River Street, Hoboken, NJ 07030, USA

Jossey-Bass, 989 Market Street, San Francisco, CA 94103-1741, USA

Wiley-VCH Verlag GmbH, Boschstr. 12, D-69469 Weinheim, Germany

John Wiley & Sons Australia Ltd, 33 Park Road, Milton, Queensland 4064, Australia

John Wiley & Sons (Asia) Pte Ltd, 2 Clementi Loop #02-01, Jin Xing Distripark, Singapore 129809

John Wiley & Sons Canada Ltd, 6045 Freemont Blvd, Mississauga, Ontario, Canada L5R 4J3

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Library of Congress Cataloging in Publication Data

The pulmonary epithelium in health and disease / edited by David Proud.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-0-470-05951-7 (cloth : alk. paper)
1. Respiratory organs—Pathophysiology. 2. Pulmonary endothelium. 3. Epithelium. I. Proud, David.
[DNLM: 1. Lung—physiopathology. 2. Epithelial Cells. 3. Epithelium—physiopathology.
WF 600 P983453 2008]
RC711.P828 2008
616.2—dc22

2008003724

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN 978-0-470-05951-7

Typeset in 10/12pt Times by Integra Software Services Pvt. Ltd, Pondicherry, India
Printed and bound in Great Britain by Antony Rowe Ltd, Chippenham, Wiltshire
This book is printed on acid-free paper.

Contents

Preface

The past two decades have seen extraordinary advances in our understanding of the role of the pulmonary epithelium in airway health and disease. The traditional view of the epithelium as predominantly a physical barrier that also plays a role in ion and water transport has been supplanted by one in which the epithelium is now also considered to be a central regulator of airway inflammation, structure and function. In light of the dramatic changes in our awareness of the complexity of epithelial cell functions, it seemed particularly timely to produce a book to comprehensively address our current understanding of epithelial cell biology. In particular, I wished to focus not only on the epithelium as a regulator of normal airway function, but also to highlight the important roles of the epithelium in host defense, and the contributions of aberrant epithelial biology to the pathogenesis of inflammatory airway diseases.

The first two chapters of this volume are designed to provide an update on the basic structure of the epithelium, including information on the cell types that comprise the epithelium at different levels of the airway, and on the capacity of specific cell types to serve as progenitor cells for new growth. In addition, the remarkable recent increases in our understanding of the molecular components of the structures that are critical for the cell-cell, and cell-matrix, adhesion necessary to maintain epithelial structure are discussed, along with the complex roles of epithelial adhesion molecules in regulating not only epithelial function but also the interactions of the epithelium with other cell types and pathogens. The subsequent two chapters focus on the role of the epithelium as a target for damage by a variety of agents, and on the process of epithelial repair. Fragility of the epithelium is a hallmark of asthma, and there is growing recognition that a chronic damage/repair cycle may play a role in the pathogenesis of this disease. Although ion transport has long been recognized as a major function of the epithelium, our understanding of the complexity and regulation of epithelial ion transport, and of the consequences of dysregulation of these events, has improved considerably in recent years, and our current knowledge is detailed in Chapter 5.

Perhaps no facet of our awareness of epithelial cell function has grown as rapidly as our understanding of the role of the epithelium in host defense, the focus of the next block of chapters. As may be expected from its location at the airway surface, the epithelium plays a critical role in protection of the host from inspired pathogens and irritants. In the larger airways, the tightly regulated process of mucociliary clearance provides the initial defense to prevent pathogens from contacting the epithelial surface, and defects in ciliary beat, or abnormal mucus composition, underlie several airway diseases that are characterized by increased susceptibility to repeated infection. In the distal airways, where mucociliary clearance is absent, surfactant plays a critical role in reducing surface tension at the airway surface. Of equal importance, however, is the role of surfactant in host defense. Not only

does it coat particulates and microbes, facilitating clearance via cough, but it is now clear that several of the protein components of surfactant have broad ranging direct antimicrobial actions. If microbes can evade these initial defenses and come into contact with the epithelium, they are detected by a range of recognition molecules. These include specific receptors as well as broad-ranging "pattern recognition molecules". Depending upon the specific nature of the ligand to be recognized, these molecules can be intracellular or expressed on the cell surface. Once microbial pattern recognition or specific receptor engagement occurs, epithelial cells respond by generating a wide range of defense molecules. These include direct antimicrobials, as well as molecules that serve to recruit and activate inflammatory cells that contribute to host defense. Finally, in this section, a major area of new investigation is the ability of the epithelium to play a major role in immunoregulation, in particular to provide an important link between innate and specific immunity.

The past decade or so also has seen marked improvements in our understanding both of the interactions of specific inhaled stimuli with the epithelium, and of the consequences of such interactions on airway function. The next set of chapters, therefore, deal with the interaction of four major classes of inhaled stimuli that affect epithelial function. Respiratory viruses not only cause upper airway diseases but also play a major role in triggering exacerbations of asthma and chronic obstructive pulmonary disease (COPD). Such effects are initiated via interactions with the epithelium. Similarly, epithelial responses to bacteria play a major pathogenic role in diseases from pneumonia, to cystic fibrosis to COPD. In our modern environment, pollutants are major exacerbators of a range of airway diseases. Finally, while the interactions of allergens with cells such as mast cells, basophils and lymphocytes obviously play a major role in allergic diseases, a growing body of literature demonstrates that interactions of allergens, particularly those with endogenous proteolytic activity, with the epithelium not only contribute to direct inflammatory effects but also play a critical role in permitting access of allergens to target cells in the underlying airway tissue.

There is now no doubt that the epithelial cell plays a major role in regulating the inflammatory and structural status of the airway. The epithelium has wide ranging synthetic and metabolic capacities. It can maintain normal airway status via its ability to inhibit or degrade a range of proinflammatory molecules but, upon repeated exposure to stimuli, can also generate a wide range of mediators that can contribute to, and exacerbate, chronic airway inflammation. Recurrent epithelial damage and repair can also cause repeated interactions between the epithelium and other structural cells, such as fibroblasts/myofibroblasts, leading to chronic reactivation of the so-called "epithelial mesenchymal trophic unit". This can lead to marked structural changes in the airway, such as the hallmark changes in asthma collectively referred to as airway remodeling.

The final set of chapters deals with the interactions of inhaled medications with the epithelium. Given the wide ranging properties discussed above, and the alterations of epithelial function in airway diseases, several of the beneficial actions of inhaled medications, including glucocorticoids, β_2-adrenergic agonists and muscarinic receptor antagonists, in diseases such as asthma and COPD may well be mediated via alterations of epithelial cell function. Last, but not least, there is growing interest in inhaled delivery of drugs, not only as a means to exert local effects in the lung, but also as a means of systemic delivery for drugs, particularly those that cannot survive oral delivery. Preserving the molecular integrity of a formulation and delivering it to the appropriate target in the lung are critical for effective therapy, and some of the recent advances in this regard are discussed in the final chapter.

Each of the chapters in this text were written by leaders in their field. Production of a text of this comprehensive nature would not have been possible without their commitment. I would like to take this opportunity to extend my sincere thanks to all of the contributors for devoting their valuable time and expertise to this volume.

David Proud

List of Contributors

Morgan Andersson
Department of Otorhinolaryngology,
Lund University Hospital
Se-221 85 Lund, Sweden

Richard C. Boucher
Cystic Fibrosis/Pulmonary Research and
Treatment Center
7011 Thurston-Bowles Building
UNC-Chapel Hill
Chapel Hill, NC 27599-7248
USA

Peter F. Bove
Cystic Fibrosis/Pulmonary Research
and Treatment Centre
The University of North Carolina
at Chapel Hill
7129 Thurston Bowles Bldg. CB#7248
Chapel Hill, NC 27599-7248, USA

Mary Mann-Jong Chang
Center for Comparative Respiratory
Biology and Medicine,
University of California at Davis,
One Shields Ave,
Davis, CA 95616, USA

Mark T. Clunes
Cystic Fibrosis/Pulmonary Research
and Treatment Centre
The University of North Carolina
at Chapel Hill
7129 Thurston Bowles Bldg. CB#7248
Chapel Hill, NC 27599-7248, USA

Louise E. Donnelly
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
Dovehouse Street
London SW3 6LY, UK

James B. Fink
Nektar Therapeutics
201 Industrial Road
San Carlos, CA 94070
USA

Marisa Gómez
Department of Pediatrics
Columbia University
650 W. 168th Street
Black Building 4-416
New York, NY 10032, USA

Wei Gong
Department of Cell Biology and Anatomy
Airway Inflammation Group
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Mark A. Giembycz
Department of Pharmacology and Therapeutics
Institute of Infection, Immunity and Inflammation
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta, Canada T2N 4N1

Catherine M. Greene
Respiratory Research Division
Royal College of Surgeons in Ireland
Education Research Centre
PO Box 9063
Beaumont Hospital
Dublin 9, Ireland

Marc B. Hershenson
University of Michigan
1150 W. Medical Center Dr.
3570 MSRBII/Box
5688 Ann Arbor
Michigan 48109-0688, USA

Pieter S. Hiemstra
Dept. of Pulmonology
Leiden University Medical Center
Albinusdreef 2
PO Box 9600
2300 RC Leiden, The Netherlands

Stephen T. Holgate
Resp. Cell & Molecular Biology Div.
Southampton General Hospital
IIR, Level D, Mail Point 810,
Centre Block
Southampton, SO16 6YD, UK

Neil S. Holden
Department of Cell Biology and Anatomy
Airway Inflammation Group
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Manminder Kaur
Department of Cell Biology and Anatomy
Airway Inflammation Group
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Elizabeth King
Department of Cell Biology and Anatomy
Airway Inflammation Group
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Richard Leigh
Departments of Medicine and of Physiology and Biophysics
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Toby C. Lewis
University of Michigan
1150 W. Medical Center Dr.
3570 MSRBII/Box
5688 Ann Arbor
Michigan 48109-0688, USA

James F. Lewis
Department of Physiology and Pharmacology
University of Western Ontario
St Josephs Health Centre, Rm. A3–003
London, Ontario, N6A 4V2 Canada

Dawn C. Newcomb
University of Michigan
1150 W. Medical Center Dr.
3570 MSRBII/Box
5688 Ann Arbor
Michigan 48109-0688, USA

Robert Newton
Dept. of Cell Biology and Anatomy
Respiratory Research Group
University of Calgary, HMRB290
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Carl G. A. Persson
Dept. of Clinical Pharmacology
Lund University Hospital
S-221 85, Lund, Sweden

Alice S. Prince
Department of Pediatrics
Columbia University
650 W. 168th Street
Black Building 4-416
New York, NY 10032, USA

David Proud
Department of Physiology and Biophysics
University of Calgary, HSC 1626
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Angela Rate
Division of Cell Biology
Telethon Institute for Child Health
Research
PO Box 855, West Perth
WA 6872 Australia

Clive Robinson
Ion Channels and Cell Signalling Centre
Division of Basic Medical Sciences
St George's, University of London
London SW17 0RE, UK

Nicole A. Rocca
The Lawson Health Research Institute
The University of Western Ontario
Department of Physiology and
Pharmacology
268 Grosvenor St., London,
ON N6A 4V2, Canada

Duncan F. Rogers
Airway Disease
National Heart and Lung Institute
Imperial College
Dovehouse Street
London, SW3 6LY, UK

Bruce K. Rubin
Department of Pediatrics
Professor of Physiology and Pharmacology
Wake Forest University School of
Medicine
Winston-Salem, NC 27157-1081 USA

Umadevi S. Sajjan
University of Michigan
1150 W. Medical Center Dr.
3570 MSRBII/Box
5688 Ann Arbor
Michigan 48109-0688, USA

Matthias Salathe
Department of Medicine, Division of
Pulmonary and Critical Care Medicine
University of Miami Miller School of
Medicine
1600 N.W. 10th Ave., RMSB 7063A
Miami, FL 33136, USA

Andreas Schmid
Department of Medicine, Division of
Pulmonary and Critical Care Medicine
University of Miami Miller School of
Medicine
1600 N.W. 10th Ave., RMSB 7063A
Miami, FL 33136, USA

Dean Sheppard
Department of Medicine
Lung Biology Center,
University of California,
1550 4th Street, San Francisco,
California 94158, USA

Laura Shih
Center for Comparative Respiratory Biology and Medicine,
University of California at Davis,
One Shields Ave,
Davis, CA 95616, USA

Donna M. Slater
Department of Pharmacology and Therapeutics
Faculty of Medicine
University of Calgary
3330 Hospital Drive NW
Calgary, Alberta T2N 4N1, Canada

Stephen G. J. Smith
Lecturer in Molecular Microbiology
Department of Clinical Microbiology
Trinity College, Trinity Centre for Health Sciences
St. James Hospital, Dublin, Ireland

Ernst Wm. Spannhake
Department of Environmental Health Sciences
Johns Hopkins Bloomberg School of Public Health
615 N. Wolfe Street
Baltimore, Maryland 21205-2103, USA

Christine Stahle
University of Michigan
1150 W. Medical Center Dr.
3570 MSRBII/Box
5688 Ann Arbor
Michigan 48109-0688
USA

Geoffrey A. Stewart
School of Biomedical
Biomolecular and Chemical Sciences
University of Western Australia
Stirling Highway, Crawley
Western Australia 6009

George Su
Department of Medicine
Division of Pulmonary and Critical Care
University of California, San Francisco
1550 4th Street, California 94158, USA

Lena Uller
Allergy and Inflammation Research
Division of Infection, Inflammation and Repair
University of Southampton, School of Medicine
Southampton General Hospital
Southampton SO16 6YD, UK

John W. Upham
School of Medicine
University of Queensland
Princess Alexandra Hospital
Brisbane, Queensland 4102
Australia

Ruud A. W. Veldhuizen
The Lawson Health Research Institute
The University of Western Ontario
Departments of Physiology and Pharmacology, and of Medicine
268 Grosvenor St., London, ON N6A 4V2
Canada

Reen Wu
University of California at Davis
Genome and Biomedical Science
451 E. Health Sciences, Ste. 6510
Davis, California 95616-5270, USA

Jihui Zhang
Ion Channels and Cell Signalling Centre
Division of Basic Medical Sciences
St George's, University of London
Cranmer Terrace
London SW17 0RE, UK

1

Pulmonary Epithelium: Cell Types and Functions

Mary Mann-Jong Chang, Laura Shih and **Reen Wu**
Center for Comparative Respiratory Biology and Medicine,
University of California at Davis,
California, USA

1.1 Introduction

The pulmonary airway tree branches in a dichotomous fashion, with repeated bifurcation stemming from the trachea. The conducting airway include the regions that do not undergo gas exchange, beginning with the trachea, which divides into two bronchi. These primary airway then branch into a series of intra-pulmonary bronchial and bronchiolar airway. Both the diameter and the length of each airway branch decrease progressively from the trachea to the periphery, where the terminal bronchioles are the most distal conducting airway (Magno and Fishman, 1982). In rodents, these bronchioles lead directly to alveolar ducts, whereas in humans and monkeys, a region of transitional respiratory bronchioles with characteristics of both bronchioles and alveoli exists between the bronchioles and the alveoli of the gas exchange area (Tyler, 1983).

The entire pulmonary tree is lined by a continuous layer of epithelial cells. The relative distribution and abundance of the epithelial cell types vary significantly, not only between species, but also within the various airway regions of each species. The pulmonary epithelium is important for maintaining the normal functions of the respiratory system, which include acting as a barrier to various insults (Widdicombe, 1987b); facilitating mucociliary clearance (Sleigh *et al*., 1988); secreting substances such as surfactant proteins, mucus, and antimicrobial peptides for airway surface protection (Widdicombe, 1987a); repairing and regenerating epithelial cells to restore normal airway function (Evans *et al*., 1976); and modulating the response of other airway components, such as airway smooth muscle cells and inflammatory cells (Flavahan *et al*., 1985; Holtzman *et al*., 1983, Breeze and Wheeldon, 1977). As many as 49 cell types have been recognized (Breeze and Wheeldon, 1977). While many of these are intermediate or differentiating cells, at least 10 to 12 morphologically and functionally unique epithelial cell types can be identified throughout the pulmonary structure (Breeze and

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

Wheeldon, 1977). They are: long and small ciliated, basal, non-ciliated secretory (goblet, Clara, surface serous, submucosal serous, and submucosal mucous), pulmonary neuroendocrine (PNE), brush, and alveolar type I and type II cell types (Figure 1.1). It is important to differentiate between these cell types, as well as to highlight the often significant species differences that may limit the experimental comparisons between various animal models and human subjects. In this chapter, we will attempt to address both of these issues while focusing on a few main mammalian systems – human, monkey, rabbit, rat, and mouse.

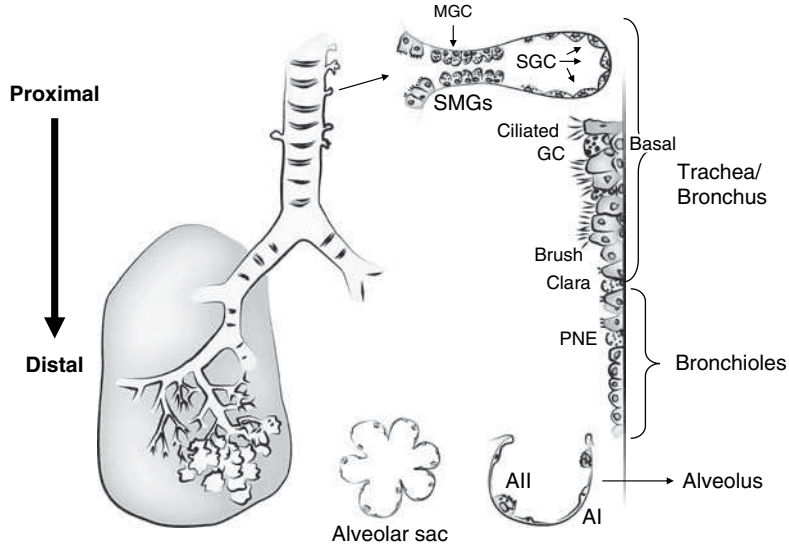


Figure 1.1 Three regions of pulmonary epithelia: cartilaginous proximal airway (trachea/bronchi and submucosal glands), non-cartilaginous distal bronchioles, and gas exchange alveoli. MGC: mucous gland cells; SGC: serous gland cells; SMGs: submucosal glands; GC: goblet cells; PNE: pulmonary neuroendocrine; AI: alveolar type I cells; AII: alveolar type II cells

The mature mammalian airway can be divided by function and structure into three regions: (1) the cartilaginous proximal airway, comprising the trachea, bronchi and submucosal glands; (2) the non-cartilaginous distal bronchioles, comprising the bronchioles, terminal bronchioles, transitional bronchioles, and respiratory bronchioles; and (3) the gas exchange region, comprising the alveolar ducts and alveolar sacs. For each region, we will discuss its epithelial makeup, the characteristic features and physiological functions of each cell type present, any known variations between species, and the role of stem and progenitor cell populations.

1.2 Epithelial cell types and functions in the cartilaginous proximal airway region

The epithelial cells of the proximal airway can be broadly separated into the surface epithelial cells of the tracheal and bronchial regions and the cells of the submucosal glands. We will first address the cell types of the tracheal and bronchial epithelium.

The epithelial cells lining the luminal surface of the proximal airway can be further grouped into ciliated cells, non-ciliated secretory cells, and basal cells. A characteristic pseudostratified two-layered epithelium persists throughout the major bronchi, while a multi-layered structure is seen in the more distal, narrow bronchi, which have fewer cartilage rings and more submucosal glands. Ciliated cells and secretory cells attach to the basal lamina via desmosome adhesions and to one another via tight junctions at the luminal surface. The underlying basal cells lie in contact with most of the basal membrane (Breeze and Wheeldon, 1977; Jeffery, 1983). Pulmonary neuroendocrine cells (PNECs) are found as single cells or in clusters throughout the proximal airway. In small animals, they are more prominent at the laryngotracheal junction and the bifurcations of intrapulmonary bronchi (Tateishi, 1973), while in humans, the PNECs are more frequently found in the smaller conducting airway (Johnson *et al*., 1982). Tracheas and bronchi from various animals reveal species-specific epithelial cell linings (Jeffery, 1983; Plopper *et al*., 1983c), with the most striking variations in the distribution of secretory cells (Plopper *et al*., 1983d).

Unique to the proximal cartilaginous airway is the existence of submucosal glands (SMGs). These glands are contiguous with the surface epithelium and are characterized by a variable proportion of ciliated cells, mucous cells and serous cells (De Poitiers *et al*., 1980). In contrast to human and monkey airway, where submucosal glands are the major secretory structure of the trachea and bronchi, SMGs in rats and mice are very scarce and limited to the upper trachea (Plopper *et al*., 1986; Widdicombe *et al*., 2001).

1.2.1 Surface epithelial cell types and functions in tracheal and bronchial regions

Ciliated cells

Ciliated cells are covered with cilia and are roughly columnar in shape, with little variation in morphological appearance between species. Ciliated cells are attached to the basal lamina via desmosomes and extend to the luminal surface, where they are interconnected via tight junctions (Rhodin, 1966). The cytoplasm of these cells is relatively electron-lucent due to their lack of secretory products or mucus granules. Many mitochondria are found in the apical region of the cell, just below the row of basal bodies to which the cilia are attached. Approximately 200–300 cilia are found on the luminal surface of each cell, with approximately half as many microvilli and fine cytoplasmic processes interspersed among them (Watson and Brinkman, 1964). In humans, the cilia are 0.25 micrometres in diameter and range from 6 micrometres in length in the proximal airway to 3.6 micrometres in seventh generation airway (Serafini and Michaelson, 1977). Their structure is comparable to that of other ciliated epithelia in plants and animals. Each cilium is anchored to the cell cytoplasm by a basal body through an axoneme. The axoneme is composed of nine microtubule doublets that formed an outer ring around a central pair of microtubules, with nexin links and radial spokes binding them together (see Chapter 6). Along each outer microtubule there are extrusions referred to as outer dynein arms (odas) and inner dynein arms (idas), both members of the dynein ATPase superfamily. Odas control the cilia beating frequency through a cAMP-dependent phosphorylation mechanism (Satir, 1999), while idas control the form of cilia beating (Brokaw and Kamiya, 1987; Friedmann and Bird, 1971). Mucociliary clearance is the major function of ciliated cells. Cilia are bathed in the watery sol phase of airway secretions and extend into the gel phase, where specialized barb-like

structures on the tips of the cilia alternatively grab and release the mucus during the active and relaxation strokes of cilia beating, thereby propelling the mucus with a rowing-like action (Jeffery and Reid, 1975).

Proliferation potential Traditionally, ciliated epithelial cells were considered to be terminally differentiated cells that did not divide, presumably originating from either basal or secretory cells (Inayama *et al*., 1989; Johnson and Hubbs, 1990). Recent reports, however, have suggested the involvement of ciliated cells in the restoration and regeneration of bronchiolar epithelium (Lawson *et al*., 2002; Park *et al*., 2006b). In the naphthalene injury model, Park *et al*. (2006b) demonstrated that ciliated cells sequentially undergo morphological transitions from squamous to cuboidal to columnar forms as the bronchiolar epithelium is restored, showing remarkable plasticity and differentiation potential. Lawson *et al*. (2002) also concluded that ciliated cells play a critical role in the repair of distal airway injury. Tyner *et al*. (2006) recently demonstrated the transdifferentiation of ciliated cells to mucous (goblet) cell metaplasia in allergic mouse airway. This transdifferentiation depends on IL-13 expression and a persistent EGFR signalling. This result further supports the theory of plasticity of ciliated airway epithelial cells. Further study is needed with isolated ciliated cells to reaffirm such a potential.

Basal cells

The ovoid basal cells form a monolayer along the basement membrane and are responsible for the pseudostratified appearance of the epithelium. Basal cells have large, indented nuclei that fill most of the cell. The cytoplasm contains many ribosomes, a small Golgi zone, a few mitochondria glycogen granules, a short profile of rough surface endoplasmic reticulum, and occasionally lysozymes. Basal cells are connected to the basement membrane through hemidesmosomes and provide the foundation for the attachment of ciliated and non-ciliated columnar cells to the basal lamina (Frasca *et al*., 1968; Breeze and Wheeldon, 1977; Rhodin, 1966). Due to their centrally located position, basal cells not only play a role in the attachment of columnar epithelium to the basement membrane, but also have the potential to function as a regulator of inflammatory response, transepithelial water movement, and oxidant defence (Evans *et al*., 2001).

Proliferation and stem cell potential One important feature of basal cells is their capacity to repopulate all the major epithelial cell types found in the trachea, including basal, ciliated, goblet and granular secretory cells (Hong *et al*., 2004b, 2004a; Inayama *et al*., 1988). Many studies have demonstrated the potential of basal cells to act in a stem cell or transient amplifying cell capacity in the upper airway. A study of 50 human bronchial biopsies with immunohistochemical staining against the proliferation agent Ki-67 revealed a population of cells that were positive for Clara cell secretory protein (CCSP) but showed no other Clara cell-specific features. This population turned out to be Ki-67 antibody-negative, but the CCSP-negative basal cells were candidate stem cells of the bronchial specimen (Barth *et al*., 2000). In another study of human trachea and bronchi using the same immunohistochemical staining, basal cells and parabasal cells composed large percentages – 51 and 33 per cent, respectively – of the proliferating compartment (Boers *et al*., 1998). Parabasal cells are located just above the basal cells and considered to be intermediate cells. The high representation of basal and parabasal cells within the proliferation compartment of normal human conducting-airway

epithelium supports the theory that cells at or near the basement membrane are likely to be the progenitor cells or transient amplifying cells of the airway surface (Hajj *et al*., 2007). In the mouse trachea, a subset of cells with high keratin 5 (K5) promoter activity residing in the submucosal gland were found to be bromodeoxyuridine label-retaining cells (LRC), which are regarded as stem cells due to their long-lasting proliferation capacity (Borthwick *et al*., 2001). Hong *et al*. (2004a) demonstrated that CCSP-expressing (CE) cells play a critical role in the renewal of bronchiolar airway. They suggested, however, that in the absence of Clara cells, basal cells may serve as secondary progenitor cells in the upper airway. Using chemically-injured mice with Clara cell ablation, they found that the cytokeratin-14 expressing basal cells were capable of restoring normal bronchial epithelium and suggested that basal cells may serve as an alternative multipotent progenitor cell in the bronchial airway (Hong *et al*., 2004b). Debate about the role of basal cells as the primary progenitors in the upper airway continues, especially since several animal injury models have shown that secretory cells, rather than basal cells, exhibit hyperproliferation after mechanical or toxic gas exposure (Johnson *et al*., 1990; Evans *et al*., 1989, Basbaum and Jany, 1990).

Non-ciliated secretory cells

The most striking interspecies difference in tracheobronchial epithelial cell types is in the distribution of non-ciliated secretory cells. In humans, ciliated cells predominate and are interspersed with mucus-secreting (goblet) cells, with approximately five ciliated cells for every goblet cell (Rhodin, 1966; Frasca *et al*., 1968). The goblet cells become less frequent in the bronchioles, as the airway becomes smaller and ciliated and Clara cells prevail (Lumsden *et al*., 1984). The major secretory cell type in sheep, monkeys, and cats is either the mucous goblet cell or the small mucous granule cell (Mariassy *et al*., 1988a; Plopper *et al*., 1989). In rats, the predominant secretory cell is the serous cell, whereas in rabbits and mice, the Clara cell is the only type of secretory cell in the entire conducting airway (Plopper *et al*., 1983a). In addition to the secretory cells of the surface epithelia, many major secretory cell types are found in the submucosal glands and will be discussed separately.

Goblet cells

Goblet cells have a relatively dense, electron-opaque cytoplasm due to the numerous mucous granules located in the apical region of the cytoplasm. The nucleus is generally compressed at the cell's basal side. The mucous granules give the cell its typical goblet shape, with a wide, enlarged apical portion and a narrow tapered basal cytoplasm. The granules in human goblet cells are electron-lucent, approximately 800 nanometres in diameter, and usually contain mucins that are acidic due to the presence of sulfate or sialic acid (Lamb and Reid, 1969; Spicer *et al*., 1971, Mariassy *et al*., 1988b).

Under healthy conditions, goblet cells, along with submucosal glands, secrete high molecular weight mucous glycoproteins that allow the surface fluid to properly trap and remove particles, thus protecting the epithelial surface. Proper regulation of mucin secretion at the airway surface is crucial to normal functioning, as overproduction can clog the airway and underproduction can impair mucociliary clearance.

Goblet (mucous) cell metaplasia in lung disease Goblet cell hyperplasia or metaplasia is a common phenomenon associated with airway inflammatory diseases, including asthma,

COPD (chronic obstructive pulmonary disease), and chronic bronchitis (Vestbo *et al*., 1996; Aikawa *et al*., 1992; Fahy, 2002; Groneberg *et al*., 2002). Goblet (or mucous) cell hyperplasia usually refers to an increase in goblet cells in the airway regions where goblet cells exist normally, such as the proximal airway of humans. Goblet (mucous) cell metaplasia, on the other hand, refers to an increase in goblet (mucous) cells in airway regions that normally contain few or no goblet cells, such as in mouse or rat airway. Both cases result in increased mucin secretion at the airway surface, thus compromising airway functions. Adler and colleagues revealed that myristoylated alanine-rich C kinase (MARCKS) is a key molecule regulating mucin exocytosis, a process also involving cooperative interaction between protein kinase C (PKC) and PKG (Park *et al*., 2006a; Singer *et al*., 2004). The use of a therapeutic agent developed in conjunction with this study may be a means of controlling mucus secretion. Using transgenic mice and an OVA-sensitized murine model, investigators have linked Th2 cytokine-mediated inflammation to goblet cell metaplasia based on studies involving IL-4, IL-9, and IL-13 (Temann *et al*., 1997; Kuperman *et al*., 2002; Vogel, 1998; Wills-Karp *et al*., 1998). Among these Th2 cytokines, IL-13 was shown to be the most potent. Studies of mice with intratracheal IL-13 instillation consistently showed increased goblet cells in the mouse airway. Additionally, goblet cell metaplasia induced by CD4 T cells and IL-9 was shown to be stimulated through a common IL-13 mediated pathway (Whittaker *et al*., 2002). Despite these findings, evidence to support IL-13 as the direct mediator of the expression of gel-forming mucin by goblet (mucous) cells is still lacking. In vivo studies may be complicated by the presence of cytokine networks and the inflammatory response upon the administration of cytokines, while in vitro studies may provide a more direct measurement of the effects of cytokines on airway epithelial cell types. Chen *et al*. (2003) have shown that IL-13 and various Th2 cytokines have no stimulatory effects on either *MUC5AC* or *MUC5B* expression in well-differentiated human airway epithelial cultures, while IL-6 and IL-17 can directly stimulate mucin gene expression. This data suggests that the transformation of airway epithelial cells into goblet cells may be a multi-step process that is controlled by different sets of cytokines.

Clara cells

For large animals such as sheep, monkeys and humans, Clara cells are concentrated in the distal conducting airway and bronchioles, while in hamsters, rabbits, and mice, the predominant non-ciliated cells throughout the entire conducting airway have the same ultrastructure features as Clara cells (Plopper *et al*., 1987; Matulionis, 1972, Jeffery and Reid, 1975). A detailed discussion of Clara cells will be presented in section 1.3, ‘Epithelial cell types and functions of the non-cartilaginous distal bronchioles’.

Surface serous cells

Serous cells on the surface airway epithelium morphologically resemble the serous cell type of the submucosal gland. They are the predominant secretory cells in rat surface epithelium (Jeffery and Reid, 1975) and have also been found sporadically in human small bronchi and bronchioles (Jeffery, 1983). In contrast to goblet and mucous cells, they have discrete electron-dense granules in the apical cytoplasm that are approximately 600 nanometres in diameter and contain neutral mucin. A detailed description of serous cell function is presented in section 1.2.2 ‘Epithelial cell types and functions in the submucosal glands’.

Pulmonary neuroendocrine cells (PNECs)

PNECs are found throughout the conducting airway of most species. They exist either individually or in clusters as neuroendocrine bodies (NEBs). In the rabbit, the NEB is a large intraepithelial organoid that is composed almost exclusively of PNECs. In other species, such as the rat, PNECs in the NEB are interspersed with Clara-like cells (Scheuermann, 1987; Sorokin *et al*., 1989; Sorokin and Hoyt, 1982). The number of PNECs and NEBs increase from the main bronchi to the terminal bronchioles, with denser populations found around bifurcating regions, such as the bronchoalveolar portals and various airway branching points (Hoyt *et al*., 1982a, 1982b). Mature PNECs are spindle-shaped, with their basal surface facing the basement membrane and a thin apical process extending toward the epithelial surface (Hage, 1980). The most prominent feature of these cells is the presence of abundant argyrophilic vesicles with granular cores concentrated at the base of the cells (Hage, 1980; Capella *et al*., 1978). As a result, PNEC secretion is polarized and directed toward adjacent cells or structures underlying the basement membrane (Hoyt *et al*., 1982a). The secretory products of the granules vary between different species and have been immunocytochemically identified as bioactive amines and peptides, including serotonin, calcitonin, gastrin-releasing peptide (GRP), calcitonin gene-related peptide (CGRP), chromogranin A, and cholecystokinin (Becker *et al*., 1980; Wharton *et al*., 1978; Sunday *et al*., 1988; Cadieux *et al*., 1986; Sirois and Cadieux, 1986). The two best-characterized peptides are GRP and the mammalian form of bombesin, CGRP. These peptides, which exert direct mitogenic effects on epithelial cells and exhibit many growth factor-like properties, are thought to be involved in normal fetal lung development, including branching morphogenesis (Li *et al*., 1994). Additionally, NEBs may play a role as hypoxia-sensitive airway chemoreceptors (Lauweryns and Cokelaere, 1973; Lauweryns *et al*., 1983) and are involved in regulating localized epithelial cell growth and regeneration (Reynolds *et al*., 2000b).

Proliferation potential PNECs are generally believed to be terminally differentiated and mitotically inert cells (Gosney, 1997). Sunday and his colleague (Sunday and Willett, 1992), however, suggested that PNEC hyperplasia in the hamster model is a result of the differentiation from proliferative stem cells or from immature PNECs. Others showed that repair from airway injury is associated with PNEC hyperplasia and that proliferation contributes to this hyperplastic response (Ito *et al*., 1994; Stevens *et al*., 1997). A study investigating the role of PNEC-derived neuropeptides in lung development suggested that PNECs are involved in the regulation of epithelial renewal (Pan *et al*., 2002). Further evidence for this theory is found in the inverse relationship between the epithelial mitotic index at each epithelial location and its distance from the closest NEB (Holt *et al*., 1990). Recently, several studies have demonstrated that NEBs provide a microenvironment for progenitor cells in the adult airway by showing that the NEB niche of normal and injured lungs supports the maintenance of at least two epithelial cell variants – one with an intermediate phenotype between Clara and PNEC cells, and the other with a Clara cell variant with little or no immuno-reactive CYP-2F2 protein (Reynolds *et al*., 2000b, 2000a). Further studies using the same naphthalene injury model demonstrated that PNECs are not stem or progenitor cells in the distal airway. Rather, they provide a niche that regulates the expansion of the CCSP-expressing stem cell population in mouse distal airway (Hong *et al*., 2001).

Brush cells

Brush cells are named for the closely packed microvilli that protrude like a brush from their luminal surface. Although they have been identified throughout the conducting airway of many species, their presence is infrequent and has not been convincingly shown in humans (Meyrick and Reid, 1968; Jeffery and Reid, 1975). While their function is not well-defined, some speculated functions include roles in periciliary fluid absorption (Jeffery, 1987), chemoreception (Luciano *et al*., 1968) and ciliogenesis (Rhodin and Dalhamn, 1956).

1.2.2 Epithelial cell types and functions in the submucosal glands

Submucosal glands are found in the upper airway of higher mammals such as humans, monkeys and sheep (Goco *et al*., 1963; Choi *et al*., 2000). They occur at a frequency of approximately one gland per square millimetre in the trachea of healthy humans and are abundant down to about the tenth generation bronchiole (Ballard *et al*., 1995). In small animals such as hamsters, rats and mice, submucosal glands are infrequently expressed and exist only in the uppermost portion of the trachea (Borthwick *et al*., 1999; Widdicombe *et al*., 2001).

Each submucosal gland consists of multiple tubules that feed into a collecting duct, which narrows into a ciliated duct that is continuous with the airway surface (Meyrick *et al*., 1969). The tubules may be inter-connecting and are lined with mucous cells in their proximal regions and serous cells in the distal acini (Meyrick *et al*., 1969). The secretory products of these two cell types are essential for proper airway mucociliary clearance. In fact, malfunctioning of serous and mucous cells may be the primary cause of many airway diseases, including chronic bronchitis, asthma, and cystic fibrosis (Salinas *et al*., 2005; Rogers, 2004; Knowles and Boucher, 2002).

Serous gland cells

Like surface serous cells, serous gland cells are pyramidal in shape, with electron-dense secretory granules in the apical region and a basally-located nucleus. The mitochondria are long and ovoid and are concentrated in the base of the cell, with a few found among the secretory granules. While most of the rough endoplasmic reticulum is at the cell base, free ribosomes are abundant throughout the cytoplasm. The Golgi apparatus is well-developed and supranuclear, often with dilated lamellae and many associated vesicles. Multivesicular bodies are also seen occasionally. Osmiophilic material is organized either into an irregularly shaped body or an irregular dense region within an electron-dense secretory granule. A large pale secretory granule containing focal condensations of osmiophilic material surrounded by a membrane is found in the apical half of most serous cells (Meyrick and Reid, 1970). Serous cells have been described as ‘immobilized neutrophils’ due to their role in the secretion of water, electrolytes, and compounds with antimicrobial, anti-inflammatory, and antioxidant properties (Basbaum *et al*., 1990). Serous cells are the predominant sites of cystic fibrosis transmembrane regulator (CFTR) expression in the human bronchus (Engelhardt *et al*., 1992a). Located distal to mucous cells, they facilitate mucociliary transport by helping remove the mucous glycoprotein produced by submucosal gland mucous cells and maintaining the airway surface liquid (ASL) volume (Inglis *et al*., 1997). CFTR malfunction in the serous cells can result in defective mucus clearance, which has been implicated as the

primary cause of cystic fibrosis (CF) disease (Knowles and Boucher, 2002; Joo *et al*., 2002; Yamaya *et al*., 1991).

Mucous gland cells

Like the surface goblet cells of the surface epithelium, mucous cells of the submucosal gland are columnar in shape, with a basally-located nucleus. The rest of the cell is packed with secretory granules of moderate electron density (Meyrick and Reid, 1970). The major function of mucous cells is to secrete mucin in the form of the mucous glycoprotein *MUC5B*, which is different from the *MUC5AC* produced by surface goblet cells (see Chapter 7). Together, these glycoproteins make up the gel phase on the apical surface of airway epithelial cells. As previously discussed in conjunction with the goblet cell, overproduction of *MUC5AC* and *MUC5B* is a common phenomenon in asthma, COPD and chronic bronchitis (Rogers, 2004, 2000; Rose *et al*., 2001).

Stem cell niche at or near submucosal glands Aside from playing a significant role in airway diseases, the submucosal gland may also provide the microenvironment for a subset of stem cells in the upper airway. Randel *et al*. discovered a high keratin-expressing subpopulation of cells residing in the submucosal gland ducts of murine trachea that were co-localized with label-retaining cells (LRCs). In mice 95 days post-injury, LRCs were localized to the gland ducts in the upper trachea and to systematically arrayed foci in the lower trachea, especially at the cartilage–intercartilage junction (Borthwick *et al*., 2001). This suggests that the submucosal gland may provide a protective niche for stem cells (Engelhardt, 2001; Borthwick *et al*., 2001).

1.3 Epithelial cell types and functions of the non-cartilaginous distal bronchioles

In most small laboratory animals such as rats, hamsters and mice, the distal bronchioles consist of several generations of non-alveolized bronchioles and a single, short alveolized bronchiole that connects to the alveolar duct. The lining epithelium is composed of simple cuboidal cells, with approximately equal numbers of ciliated cells and non-ciliated Clara cells (Widdicombe and Pack, 1982; Plopper *et al*., 1983b). In higher mammals such as humans and monkeys, however, there are several generations of both non-alveolized and alveolized (respiratory) bronchioles (Castleman *et al*., 1975; Tyler, 1983). The non-alveolized bronchioles are lined with ciliated cells and non-ciliated secretory cells, while the alveolized bronchioles are scattered with alveolar type I and type II cells amongst simple cuboidal cells.

Clara cells

Although there are significant inter- and intra-species variations in their ultrastructural characteristics, Clara cells are generally ovoid or columnar in shape, with a centrally-located nucleus, prominent Golgi, and abundant organelles including agranular and granular endoplasmic reticulum. Their most prominent features are the membrane-bound electron-dense secretory granules. While the granules do not contain glycoprotein, Clara cells are metabolically active. CC10 (or CCSP) is a secreted protein homologous to uteroglobin

that may be important in regulating the inflammatory response and is used as a Clara cell marker (Plopper *et al*., 1980c, 1980a, 1980b; Widdicombe and Pack, 1982; Singh *et al*., 1990). The surfactant protein SP-B is another secretory product of Clara cells that may be involved in host defence activity (Phelps and Floros, 1991). These cells also produce proteins with inhibitory effects on proteases; one such example is the antileukoproteases found one the surface of human airway (Simionescu and Simionescu, 1983; Yoneda and Walzer, 1984). Furthermore, Clara cells have the capacity to metabolize xenobiotics through their cytochrome p450 monooxygenase activity, a function that renders them susceptible to injury by lipophilic compounds (Baron *et al*., 1988).

Stem cell niche at the bronchioalveolar region The most important property of Clara cells is their ability to act as stem cells. Clara cells have long been considered to be progenitor cells for the terminal bronchioles (Evans *et al*., 1976, 1978). Repopulation studies of specific epithelial cell types in vitro and in vivo suggested that basal cells and bronchiolar Clara cells have stem and progenitor cell capabilities in the regeneration of the trachea, bronchi, and bronchioles (Nettesheim *et al*., 1990). In the study of normal human lungs obtained from autopsy, triple sequential histochemical staining was used to elucidate the contribution of Clara cells to the proliferation compartment. Using MIB-1 as a proliferation marker, anti-CC10 for the identification of Clara cells, and a PAS stain marker for goblet cells, Clara cells were found to be absent in the proximal airway epithelium, while their contribution to the proliferation compartment in the respiratory bronchioles was 44 per cent. This demonstrated that Clara cells play an important role in the normal maintenance of the human distal conducting airway epithelium (Boers *et al*., 1999). Recent studies using naphthalene-injured mice have suggested that a subset of naphthalene-resistant Clara cells in the bronchiolar epithelium acts as a stem cell population. In mice whose Clara cells were ablated by naphthalene, a population of variant Clara cells that were cytochrome p450 2F2 negative and resided in discrete pools associated with neuroepithelial bodies (NEBs) were found to exhibit multipotent differentiation and to regenerate the bronchiolar epithelium (Reynolds *et al*., 2000a, 2000b). The associated neuroendocrine cells are thought to provide a niche that regulates the expansion of Clara cell secretory protein (CCSP)-expressing cells (Hong *et al*., 2001). In a study searching for cells contributing to the renewal of terminal bronchioles after Clara cell depletion in mice, CCSP-expressing cells that were localized to the bronchioalveolar duct junction (BADJ) were also identified as the predominant proliferative population in initial terminal bronchiolar repair. These cells included a population of label-retaining cells, characteristic of a stem cell population. Furthermore, immunohistochemical co-localization studies involving CCSP and the NEB-specific marker, calcitonin gene-related peptide, indicate that BADJ-associated CCSP-expressing stem cells function independently of NEB microenvironments. These studies identify a BADJ-associated, NEB-independent, CCSP-expressing stem cell population in terminal bronchioles and support the theory that region-specific stem cell niches exist to maintain epithelial diversity after injury (Giangreco *et al*., 2002). Identified at the bronchioalveolar duct junction, bronchioalveolar stem cells (BASCs) retain characteristics of regional stem cells such as LRC accumulation, self-renewal, and multipotency in clonal assays. BASCs are believed to maintain the Clara cell and alveolar cell populations in the distal airway. Interestingly, Clara cells and alveolar cells of the distal lung and their transformed counterparts give rise to adenocarcinoma. This work also points to BASCs as the putative origin cells for this subtype of lung cancer (Kim *et al*., 2005).

1.4 Epithelial cell types and functions of the gas exchange region

The main function of the pulmonary acini is to facilitate efficient gas exchange between blood and air. The air–blood barrier is a three-layered structure consisting of capillary endothelium, basement membrane, and a thin, membrane-like epithelium that allows diffusion of gases while serving as a barrier against the leakage of solutions into the alveoli (Gehr *et al*., 1978). This thin layer of epithelium is composed of large, flat alveolar type I cells that cover 90 per cent of the alveolar surface, and cuboidal alveolar type II cells that cover the remaining 10 per cent (Haies *et al*., 1981). Tight junctions form a gasket-like seal between adjoining cells and help maintain their structural and functional polarity (Schneeberger and Hamelin, 1984).

Alveolar type I cells

Alveolar type I cells are large, flat squamous cells with a relatively simple structure that function mostly as a thin, gas-permeable membrane. Each cell has a small nucleus surrounded by a few small mitochondria, an inconspicuous Golgi apparatus, and some cisternae of endoplasmic reticulum with ribosomes (Low, 1952). There are also pinocytotic vesicles in the peripheral region of the cytoplasm and at both the alveolar and interstitial surfaces of the cells (Gil *et al*., 1981). The vesicles are thought to be involved in protein transportation between cells and alveoli (Bignon *et al*., 1976; Schneeberger and Hamelin, 1984).

Proliferation potential Alveolar type I cells are sensitive to injury by various agents, such as NO_2 (Evans *et al*., 1975), ozone (Plopper *et al*., 1973), and bleomycin (Jones and Reeve, 1978). If the damage is lethal, the cells detach, exposing denuded basement membrane. Alveolar type I cells are considered to be terminally differentiated and cannot divide; therefore, they must depend on the mitosis and differentiation of alveolar type II cells for repopulation (Evans *et al*., 1975).

Alveolar type II cells

Alveolar type II cells are small and cuboidal in shape, and constitute approximately 15 per cent of the cells of the alveolar epithelium. They contain unique lamellar bodies and various organelles, including mitochondria, endoplasmic reticulum, filaments, microtubules, and pinocytic vesicles (Macklin, 1954; Crapo *et al*., 1982). The cells are structurally and functionally polarized due to the existence of tight junctions at the lateral cell surface that divide the cell into apical and basolateral domains. The apical membrane contains molecules not found in the basolateral membrane, such as glycoprotein 330 (Chatelet *et al*., 1986), alkaline phosphatase (Edelson *et al*., 1988), and special glycosylated molecules recognized by lectin. The apical cell membrane also has numerous short microvilli, which are used to identify type II cells (Wright *et al*., 1986). Secretion and endocytosis take place mostly in the apical domain.

The most important function of alveolar type II cells is the synthesis and secretion of surface-active materials, referred to as surfactants (see Chapter 8). Pulmonary surfactants

are a complex mixture of proteins and phospholipids that lower surface tension at the air–liquid interface and prevent the alveolar surface from collapsing (Wright and Dobbs, 1991; Dobbs, 1994). They consist predominantly of phospholipids that are rich in dipalmitoylphosphatidylcholine and phosphatidylglycerol synthesized by type II cells, along with several unique proteins such as surfactant proteins SP-A, SP-B, SP-C and SP-D (Rooney *et al*., 1994; Batenburg and Haagsman, 1998). The appropriate composition of pulmonary surfactants is crucial to normal functioning. For example, a deficiency of dipalmitoylphosphatidylcholine at the alveolar surface has been associated with infant respiratory distress syndrome (RDS). Prior to secretion, the surfactants are stored in lamellar bodies as densely packed lamellae and are secreted into the alveolar lumen by regulated exocytosis. In this process, lamellar bodies are propelled to the apex, where they fuse with the membrane and release their contents into the alveolus (Ryan *et al*., 1975). After the surfactant lipids are released, the spheroid lamellar bodies reorganize into an expanded membrane lattice called 'tubular myelin' (Williams and Mason, 1977). Alveolar type II cells can also endocytose surfactant from the alveolar space via small pinocytic membrane-bound vesicles that form multivesicular bodies involved in endocytic transportation. The materials taken up by this pathway are largely recycled to lamellar bodies (Williams, 1984; Hallman and Teramo, 1981; Chander *et al*., 1987), with remaining materials degraded (Chander *et al.,* 1987).

Proliferation potential and stem cell niche in alveoli Alveolar type II cells are believed to be the only stem cell of the alveolar epithelium, able to proliferate as well as differentiate into alveolar type I cells (Mason *et al*., 1997; Griffiths *et al*., 2005; Reynolds *et al*., 2004; Gomperts and Strieter, 2007; Uhal, 1997; Weiss *et al*., 2006). Numerous in vivo animal studies have demonstrated the ability of type II cells to repopulate the alveolar epithelium. Briefly, various pollutants and reagents were used to injure the airway epithelium (Liu *et al*., 2006). Following the injury event, type II cells were observed to proliferate and differentiate into type I cells to restore the alveolar epithelium, with cells showing characteristics of both alveolar types in the intermediate stages (Evans *et al*., 1973, 1975, 1972; Kapanci *et al*., 1969; Adamson and Bowden, 1974, 1975; Aso *et al*., 1976). The ability of alveolar type II cells to differentiate into type I cells has also been demonstrated in vitro. Type II cells isolated from rats begin to exhibit type I cell characteristics after a period of in vitro culture (Brody and Williams, 1992; Danto *et al*., 1992, Dobbs *et al*., 1988; Kikkawa and Yoneda, 1974; Paine *et al*., 1988; Paine and Simon, 1996). Altering the culture substrate has an effect on whether type II cells retain their characteristics or differentiate into type I cells, highlighting the importance of the extracellular matrix microenvironment in determining cell fate (Shannon *et al*., 1992).

Type II cells themselves are a heterogeneous group. Studies have shown that some type II cells are more susceptible to injury than others, and the true stem cell population within the group has been characterized as E-cadherin negative, proliferative, and having high telomerase expression (Adamson and Bowden, 1975; Reddy *et al*., 2004). Though much less prevalent in the literature, there is also evidence that alveolar type I cells differentiated from type II cells can dedifferentiate back into type II cells under certain conditions (Danto *et al*., 1995). This may lead to the classification of type I cells as a limited progenitor cell as well, although there is a general consensus that type II cells are the stem cells of the alveolar epithelium.

1.5 Circulating stem cells and applications in lung regenerative medicine

Many reports have suggested that adult bone marrow acts as a source of circulating stem cells that localize to various tissues and differentiate into tissue-specific cells (Anjos-Afonso *et al*., 2004; Herzog *et al*., 2003; Jiang *et al*., 2002; Korbling and Estrov, 2003; Neuringer and Randell, 2004; Pereira *et al*., 1995; Prockop, 2003; Wagers *et al*., 2002). Multiple subpopulations of bone marrow may be involved, including haematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, fibrocytes, and circulating epithelial progenitor cells (Direkze *et al*., 2003; Schmidt *et al*., 2003; Bucala *et al*., 1994; Epperly *et al*., 2003; Hashimoto *et al*., 2004; Kotton *et al*., 2001; Krause *et al*., 2001). Most of the evidence comes from animal and clinical transplant cases, which arguably revealed chimerism and engraftment of donor cells. In multiple studies involving bone marrow transplants in animals, donor bone marrow-derived cells were identified in the lung with lung cell phenotypes (Abe *et al*., 2004, 2003; Anjos-Afonso *et al*., 2004; Beckett *et al*., 2005; Epperly *et al*., 2003; Grove *et al*., 2002; Hashimoto *et al*., 2004; Jiang *et al*., 2002; Kotton *et al*., 2001; Krause *et al*., 2001; Loi *et al*., 2006; Macpherson *et al*., 2005; Ortiz *et al*., 2003; Pereira *et al*., 1995; Rojas *et al*., 2005; Schoeberlein *et al*., 2005; Theise *et al*., 2002; Yamada *et al*., 2004). In human bone marrow transplants, chimerism of epithelial and endothelial cells as well as engraftment of bone marrow-derived cells were found in lung tissue (Mattsson *et al*., 2004; Suratt *et al*., 2003; Albera *et al*., 2005). Furthermore, chimerism and engraftment have also appeared in the lung epithelium following human lung transplants, suggesting that circulating stem cells in the recipient can localize to the donor lung (Kleeberger *et al*., 2003; Spencer *et al*., 2005; Albera *et al*., 2005).

There is also evidence that bone marrow-derived cells localize to sites of lung injury and help mitigate the damage (Abe *et al*., 2004; Epperly *et al*., 2003, Gomperts *et al*., 2006; Hashimoto *et al*., 2004; Ishizawa *et al*., 2004; Kotton *et al*., 2001; Ortiz *et al*., 2003; Rojas *et al*., 2005; Theise *et al*., 2002; Yamada *et al*., 2004, 2005; Ishii *et al*., 2005; Moore *et al*., 2005; Burnham *et al*., 2005). Other studies, however, have suggested that in some cases, bone marrow-derived cells may actually contribute to fibrosis (Epperly *et al*., 2003; Hashimoto *et al*., 2004; Phillips *et al*., 2004). Indeed, controversy remains about the actual ameliorative effect of circulating stem cells, whether or not they can engraft in other organs, and whether engrafted cells undergo fusion or transdifferentiation (Aliotta *et al*., 2005; Vassilopoulos *et al*., 2003; Wang *et al*., 2003; Chang *et al*., 2005; Davies *et al*., 2002; Kotton *et al*., 2005; Zander *et al*., 2005; Loi *et al*., 2006). Clearly, researchers have not yet reached a consensus about the role that circulating stem cells play in lung processes.

1.6 Stem cell therapy: embryonic or adult?

Stem cell therapy has been vaunted as a possible source of cures. We hope that stem or progenitor cells can be used to repair injury and fix diseases, or that an endogenous stem cell population can be targeted for gene therapy. While stem cell therapies using embryonic stem cells or endogenous stem cells of the pulmonary system have thus far been limited to speculation, some studies have shown that bone marrow-derived stem cells may have an ameliorative effect on lung diseases and injuries (Abe *et al*., 2004; Ishizawa *et al*.,

2004; Ortiz *et al*., 2003; Rojas *et al*., 2005; Yamada *et al*., 2004, 2005; Burnham *et al*., 2005; Gomperts *et al*., 2006). As previously discussed, much debate continues over the therapeutic effects of these circulating stem cells. The cell subpopulation most appropriate for therapeutic application remains to be identified, and their in vivo proliferation and differentiation activity defined. As seen in cases where applied bone marrow-derived stem cells can actually contribute to a disease state (Epperly *et al*., 2003; Hashimoto *et al*., 2004; Phillips *et al*., 2004), great care must be taken when introducing stem cells into the system. Though embryonic stem cells have not yet been used in cell therapy for the pulmonary system, researchers have had moderate success in obtaining airway epithelial cells from mouse and human embryonic stem cells (Ali *et al*., 2002; Coraux *et al*., 2005; Nishimura *et al*., 2004, 2006; Rippon *et al*., 2004, 2006; Samadikuchaksaraei *et al*., 2006; Wang *et al*., 2007). Although functional pulmonary epithelial cells differentiated from embryonic stem cells might one day be useful in treating disease, immunological difficulties could prove to be the biggest obstacle to overcome. Until these problems are solved, the embryonic stem cell system may contribute mostly to the areas of understanding developmental and disease processes. The endogenous stem cells of the lung present another potential pool of cells for transplantation or gene therapy, but the definitive characterization of these stem cell populations must first be completed. Additionally, the ability to isolate pure populations of these cells could enhance current xenograft models of airway epithelium regeneration, which have demonstrated the ability of airway epithelial cells to repopulate a denuded trachea (Puchelle and Peault, 2000; Shimizu *et al*., 1994; Engelhardt *et al*., 1992b, 1995; Zepeda *et al*., 1995; Dupuit *et al*., 2000; Castillon *et al*., 2004; Escotte *et al*., 2004). Using this technique in a more limited, well-controlled manner alongside gene therapy techniques could offer new treatments using a patient's own pulmonary stem cells – perhaps altered or enhanced in vitro – to treat airway epithelial diseases and injuries (Castillon *et al*., 2004; Engelhardt *et al*., 1992b).

Another area that requires further study for all stem cell populations is the stem cell niche, or microenvironment. We must fully understand the effects that the microenvironment has on stem cell proliferation and differentiation before we can be confident of the safety and efficacy of any stem cell therapy. While some soluble factors have been studied – especially in areas of embryogenesis and development – researchers have only begun to understand their effects and those of the three-dimensional extracellular matrix (Warburton *et al*., 2005; Dunsmore and Rannels, 1996). With further study, pulmonary diseases may one day be treated with the help of stem cells.

1.7 Conclusion

In addition to facilitating the exchange of respiratory gases, the pulmonary epithelium is a physical barrier that is constantly exposed to infectious organisms, oxidative stress, and toxins from the external environment. Roughly 10 to 12 epithelial cell types can be identified in the pulmonary epithelium. The distribution of these epithelial cell types is species-dependent and airway region-specific (Figure 1.1). Roughly, the distribution is correlated to the functions of each airway segment. In the trachea and bronchi, these functions are the trapping and removal of particles and infectious microorganisms. To perform these functions, ciliated, basal and non-ciliated secretory cells capable of mucus secretion are predominately present. In the distal bronchioles, only minimal mucociliary function is undertaken in the narrowing airway

space. The major function in this distal region is to sense and condition the incoming air, requiring mainly Clara and PNE cells. Among Clara cells, there are differences in cytochrome p450-mediated drug metabolism as well as local distribution. In the gas exchange region, alveolar type I cells contribute a large cell surface area, while cuboidal type II cells are responsible for surfactant production to prevent lung collapse. To maintain airway integrity and efficiently respond to injury, the pulmonary epithelia should contain active stem cell niches throughout the airway that can immediately produce transient amplifying cells when needed. There have been extensive studies to identify these niches and the specific cell type(s) serving as adult stem cells. These studies may one day lead to the development of cell therapies for various airway and lung diseases.

References

Abe, S., Boyer, C., Liu, X., Wen, F. Q., Kobayashi, T., Fang, Q., Wang, X., Hashimoto, M., Sharp, J. G. & Rennard, S. I. (2004) Cells derived from the circulation contribute to the repair of lung injury. *Am J Respir Crit Care Med*, **170**, 1158–63.

Abe, S., Lauby, G., Boyer, C., Rennard, S. I. & Sharp, J. G. (2003) Transplanted BM and BM side population cells contribute progeny to the lung and liver in irradiated mice. *Cytotherapy*, **5**, 523–33.

Adamson, I. Y. & Bowden, D. H. (1974) The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. *Lab Invest*, **30**, 35–42.

Adamson, I. Y. & Bowden, D. H. (1975) Derivation of type 1 epithelium from type 2 cells in the developing rat lung. *Lab Invest*, **32**, 736–45.

Aikawa, T., Shimura, S., Sasaki, H., Ebina, M. & Takishima, T. (1992) Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest*, **101**, 916–21.

Albera, C., Polak, J. M., Janes, S., Griffiths, M. J., Alison, M. R., Wright, N. A., Navaratnarasah, S., Poulsom, R., Jeffery, R., Fisher, C., Burke, M. & Bishop, A. E. (2005) Repopulation of human pulmonary epithelium by bone marrow cells: a potential means to promote repair. *Tissue Eng*, **11**, 1115–21.

Ali, N. N., Edgar, A. J., Samadikuchaksaraei, A., Timson, C. M., Romanska, H. M., Polak, J. M. & Bishop, A. E. (2002) Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng*, **8**, 541–50.

Aliotta, J. M., Passero, M., Meharg, J., Klinger, J., Dooner, M. S., Pimentel, J. & Quesenberry, P. J. (2005) Stem cells and pulmonary metamorphosis: new concepts in repair and regeneration. *J Cell Physiol*, **204**, 725–41.

Anjos-Afonso, F., Siapati, E. K. & Bonnet, D. (2004) In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. *J Cell Sci*, **117**, 5655–64.

Aso, Y., Yoneda, K. & Kikkawa, Y. (1976) Morphologic and biochemical study of pulmonary changes induced by bleomycin in mice. *Lab Invest*, **35**, 558–68.

Ballard, S. T., Fountain, J. D., Inglis, S. K., Corboz, M. R. & Taylor, A. E. (1995) Chloride secretion across distal airway epithelium: relationship to submucosal gland distribution. *Am J Physiol*, **268**, L526–31.

Baron, J., Burke, J. P., Guengerich, F. P., Jakoby, W. B. & Voigt, J. M. (1988) Sites for xenobiotic activation and detoxication within the respiratory tract: implications for chemically induced toxicity. *Toxicol Appl Pharmacol*, **93**, 493–505.

Barth, P. J., Koch, S., Muller, B., Unterstab, F., Von Wichert, P. & Moll, R. (2000) Proliferation and number of Clara cell 10-kDa protein (CC10)-reactive epithelial cells and basal cells in normal, hyperplastic and metaplastic bronchial mucosa. *Virchows Arch*, **437**, 648–55.

Basbaum, C. & Jany, B. (1990) Plasticity in the airway epithelium. *Am J Physiol*, **259**, L38–46.

Basbaum, C. B., Jany, B. & Finkbeiner, W. E. (1990) The serous cell. *Annu Rev Physiol*, **52**, 97–113.
Batenburg, J. J. & Haagsman, H. P. (1998) The lipids of pulmonary surfactant: dynamics and interactions with proteins. *Prog Lipid Res*, **37**, 235–76.
Becker, K. L., Monaghan, K. G. & Silva, O. L. (1980) Immunocytochemical localization of calcitonin in Kulchitsky cells of human lung. *Arch Pathol Lab Med*, **104**, 196–8.
Beckett, T., Loi, R., Prenovitz, R., Poynter, M., Goncz, K. K., Suratt, B. T. & Weiss, D. J. (2005) Acute lung injury with endotoxin or NO_2 does not enhance development of airway epithelium from bone marrow. *Mol Ther*, **12**, 680–6.
Bignon, J., Jaurand, M. C., Pinchon, M. C., Sapin, C. & Warnet, J. M. (1976) Immunoelectron microscopic and immunochemical demonstrations of serum proteins in the alveolar lining material of the rat lung. *Am Rev Respir Dis*, **113**, 109–20.
Boers, J. E., Ambergen, A. W. & Thunnissen, F. B. (1998) Number and proliferation of basal and parabasal cells in normal human airway epithelium. *Am J Respir Crit Care Med*, **157**, 2000–6.
Boers, J. E., Ambergen, A. W. & Thunnissen, F. B. (1999) Number and proliferation of clara cells in normal human airway epithelium. *Am J Respir Crit Care Med*, **159**, 1585–91.
Borthwick, D. W., West, J. D., Keighren, M. A., Flockhart, J. H., Innes, B. A. & Dorin, J. R. (1999) Murine submucosal glands are clonally derived and show a cystic fibrosis gene-dependent distribution pattern. *Am J Respir Cell Mol Biol*, **20**, 1181–9.
Borthwick, D. W., Shahbazian, M., Krantz, Q. T., Dorin, J. R. & Randell, S. H. (2001) Evidence for stem-cell niches in the tracheal epithelium. *Am J Respir Cell Mol Biol*, **24**, 662–70.
Breeze, R. G. & Wheeldon, E. B. (1977) The cells of the pulmonary airways. *Am Rev Respir Dis*, **116**, 705–77.
Brody, J. S. & Williams, M. C. (1992) Pulmonary alveolar epithelial cell differentiation. *Annu Rev Physiol*, **54**, 351–71.
Brokaw, C. J. & Kamiya, R. (1987) Bending patterns of *Chlamydomonas flagella*: IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. *Cell Motil Cytoskeleton*, **8**, 68–75.
Bucala, R., Spiegel, L. A., Chesney, J., Hogan, M. & Cerami, A. (1994) Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med*, **1**, 71–81.
Burnham, E. L., Taylor, W. R., Quyyumi, A. A., Rojas, M., Brigham, K. L. & Moss, M. (2005) Increased circulating endothelial progenitor cells are associated with survival in acute lung injury. *Am J Respir Crit Care Med*, **172**, 854–60.
Cadieux, A., Springall, D. R., Mulderry, P. K., Rodrigo, J., Ghatei, M. A., Terenghi, G., Bloom, S. R. & Polak, J. M. (1986) Occurrence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of capsaicin treatment and surgical denervations. *Neuroscience*, **19**, 605–27.
Capella, C., Hage, E., Solcia, E. & Usellini, L. (1978) Ultrastructural similarity of endocrine-like cells of the human lung and some related cells of the gut. *Cell Tissue Res*, **186**, 25–37.
Castillon, N., Avril-Delplanque, A., Coraux, C., Delenda, C., Peault, B., Danos, O. & Puchelle, E. (2004) Regeneration of a well-differentiated human airway surface epithelium by spheroid and lentivirus vector-transduced airway cells. *J Gene Med*, **6**, 846–56.
Castleman, W. L., Dungworth, D. L. & Tyler, W. S. (1975) Intrapulmonary airway morphology in three species of monkeys: a correlated scanning and transmission electron microscopic study. *Am J Anat*, **142**, 107–21.
Chander, A., Reicherter, J. & Fisher, A. B. (1987) Degradation of dipalmitoyl phosphatidylcholine by isolated rat granular pneumocytes and reutilization for surfactant synthesis. *J Clin Invest*, **79**, 1133–8.
Chang, J. C., Summer, R., Sun, X., Fitzsimmons, K. & Fine, A. (2005) Evidence that bone marrow cells do not contribute to the alveolar epithelium. *Am J Respir Cell Mol Biol*, **33**, 335–42.
Chatelet, F., Brianti, E., Ronco, P., Roland, J. & Verroust, P. (1986) Ultrastructural localization by monoclonal antibodies of brush border antigens expressed by glomeruli. I. Renal distribution. *Am J Pathol*, **122**, 500–11.

Chen, Y., Thai, P., Zhao, Y. H., Ho, Y. S., Desouza, M. M. & Wu, R. (2003) Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem*, **278**, 17036–43.

Choi, J. A., Kim, J. H., Hong, K. T., Kim, H. S., Oh, Y. W. & Kang, E. Y. (2000) CT bronchus sign in malignant solitary pulmonary lesions: value in the prediction of cell type. *Eur Radiol*, **10**, 1304–9.

Coraux, C., Nawrocki-Raby, B., Hinnrasky, J., Kileztky, C., Gaillard, D., Dani, C. & Puchelle, E. (2005) Embryonic stem cells generate airway epithelial tissue. *Am J Respir Cell Mol Biol*, **32**, 87–92.

Crapo, J. D., Barry, B. E., Gehr, P., Bachofen, M. & Weibel, E. R. (1982) Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis*, **126**, 332–7.

Danto, S. I., Zabski, S. M. & Crandall, E. D. (1992) Reactivity of alveolar epithelial cells in primary culture with type I cell monoclonal antibodies. *Am J Respir Cell Mol Biol*, **6**, 296–306.

Danto, S. I., Shannon, J. M., Borok, Z., Zabski, S. M. & Crandall, E. D. (1995) Reversible transdifferentiation of alveolar epithelial cells. *Am J Respir Cell Mol Biol*, **12**, 497–502.

Davies, J. C., Potter, M., Bush, A., Rosenthal, M., Geddes, D. M. & Alton, E. W. (2002) Bone marrow stem cells do not repopulate the healthy upper respiratory tract. *Pediatr Pulmonol*, **34**, 251–6.

De Poitiers, W., Lord, P. W., Biles, B. & Whimster, W. F. (1980) Bronchial gland histochemistry in lungs removed for cancer. *Thorax*, **35**, 546–51.

Direkze, N. C., Forbes, S. J., Brittan, M., Hunt, T., Jeffery, R., Preston, S. L., Poulsom, R., Hodivala-Dilke, K., Alison, M. R. & Wright, N. A. (2003) Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. *Stem Cells*, **21**, 514–20.

Dobbs, L. G. (1994) Alveoli and airways: possible interactions in the pathogenesis of asthma. *Am J Respir Crit Care Med*, **150**, S31–2.

Dobbs, L. G., Williams, M. C. & Gonzalez, R. (1988) Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim Biophys Acta*, **970**, 146–56.

Dunsmore, S. E. & Rannels, D. E. (1996) Extracellular matrix biology in the lung. *Am J Physiol*, **270**, L3–27.

Dupuit, F., Gaillard, D., Hinnrasky, J., Mongodin, E., De Bentzmann, S., Copreni, E. & Puchelle, E. (2000) Differentiated and functional human airway epithelium regeneration in tracheal xenografts. *Am J Physiol Lung Cell Mol Physiol*, **278**, L165–76.

Edelson, J. D., Shannon, J. M. & Mason, R. J. (1988) Alkaline phosphatase: a marker of alveolar type II cell differentiation. *Am Rev Respir Dis*, **138**, 1268–75.

Engelhardt, J. F. (2001) Stem cell niches in the mouse airway. *Am J Respir Cell Mol Biol*, **24**, 649–52.

Engelhardt, J. F., Yankaskas, J. R., Ernst, S. A., Yang, Y., Marino, C. R., Boucher, R. C., Cohn, J. A. & Wilson, J. M. (1992a) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nat Genet*, **2**, 240–8.

Engelhardt, J. F., Yankaskas, J. R. & Wilson, J. M. (1992b) In vivo retroviral gene transfer into human bronchial epithelia of xenografts. *J Clin Invest*, **90**, 2598–607.

Engelhardt, J. F., Schlossberg, H., Yankaskas, J. R. & Dudus, L. (1995) Progenitor cells of the adult human airway involved in submucosal gland development. *Development*, **121**, 2031–46.

Epperly, M. W., Guo, H., Gretton, J. E. & Greenberger, J. S. (2003) Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. *Am J Respir Cell Mol Biol*, **29**, 213–24.

Escotte, S., Catusse, C., Coraux, C. & Puchelle, E. (2004) Reconstitution of human airway tissue in the humanized xenograft model. *J Cyst Fibros*, **3** Suppl 2, 63–5.

Evans, M. J., Stephens, R. J., Cabral, L. J. & Freeman, G. (1972) Cell renewal in the lungs of rats exposed to low levels of NO_2. *Arch Environ Health*, **24**, 180–8.

Evans, M. J., Cabral, L. J., Stephens, R. J. & Freeman, G. (1973) Renewal of alveolar epithelium in the rat following exposure to NO_2. *Am J Pathol*, **70**, 175–98.

Evans, M. J., Cabral, L. J., Stephens, R. J. & Freeman, G. (1975) Transformation of alveolar type 2 cells to type 1 cells following exposure to NO_2. *Exp Mol Pathol*, **22**, 142–50.

Evans, M. J., Johnson, L. V., Stephens, R. J. & Freeman, G. (1976) Renewal of the terminal bronchiolar epithelium in the rat following exposure to NO_2 or O_3. *Lab Invest*, **35**, 246–57.

Evans, M. J., Cabral-Anderson, L. J. & Freeman, G. (1978) Role of the Clara cell in renewal of the bronchiolar epithelium. *Lab Invest*, **38**, 648–53.

Evans, M. J., Cox, R. A., Shami, S. G., Wilson, B. & Plopper, C. G. (1989) The role of basal cells in attachment of columnar cells to the basal lamina of the trachea. *Am J Respir Cell Mol Biol*, **1**, 463–9.

Evans, M. J., Van Winkle, L. S., Fanucchi, M. V. & Plopper, C. G. (2001) Cellular and molecular characteristics of basal cells in airway epithelium. *Exp Lung Res*, **27**, 401–15.

Fahy, J. V. (2002) Goblet cell and mucin gene abnormalities in asthma. *Chest*, **122**, 320S–326S.

Flavahan, N. A., Aarhus, L. L., Rimele, T. J. & Vanhoutte, P. M. (1985) Respiratory epithelium inhibits bronchial smooth muscle tone. *J Appl Physiol*, **58**, 834–8.

Frasca, J. M., Auerbach, O., Parks, V. R. & Jamieson, J. D. (1968) Electron microscopic observations of the bronchial epithelium of dogs. II. Smoking dogs. *Exp Mol Pathol*, **9**, 380–99.

Friedmann, I. & Bird, E. S. (1971) Ciliary structure, ciliogenesis, microvilli. (Electron microscopy of the mucosa of the upper respiratory tract). *Laryngoscope*, **81**, 1852–68.

Gehr, P., Bachofen, M. & Weibel, E. R. (1978) The normal human lung: ultrastructure and morphometric estimation of diffusion capacity. *Respir Physiol*, **32**, 121–40.

Giangreco, A., Reynolds, S. D. & Stripp, B. R. (2002) Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am J Pathol*, **161**, 173–82.

Gil, J., Silage, D. A. & McNiff, J. M. (1981) Distribution of vesicles in cells of air-blood barrier in the rabbit. *J Appl Physiol*, **50**, 334–40.

Goco, R. V., Kress, M. B. & Brantigan, O. C. (1963) Comparison of mucus glands in the tracheobronchial tree of man and animals. *Ann N Y Acad Sci*, **106**, 555–71.

Gomperts, B. N. & Strieter, R. M. (2007) *Annu Rev Med*, **58**, 285–298.

Gomperts, B. N., Belperio, J. A., Rao, P. N., Randell, S. H., Fishbein, M. C., Burdick, M. D. & Strieter, R. M. (2006) Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J Immunol*, **176**, 1916–27.

Gosney, J. R. (1997) Pulmonary neuroendocrine cell system in pediatric and adult lung disease. *Microsc Res Tech*, **37**, 107–13.

Griffiths, M. J., Bonnet, D. & Janes, S. M. (2005) Stem cells of the alveolar epithelium. *Lancet*, **366**, 249–60.

Groneberg, D. A., Eynott, P. R., Oates, T., Lim, S., Wu, R., Carlstedt, I., Nicholson, A. G. & Chung, K. F. (2002) Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. *Respir Med*, **96**, 81–6.

Grove, J. E., Lutzko, C., Priller, J., Henegariu, O., Theise, N. D., Kohn, D. B. & Krause, D. S. (2002) Marrow-derived cells as vehicles for delivery of gene therapy to pulmonary epithelium. *Am J Respir Cell Mol Biol*, **27**, 645–51.

Hage, E. (1980) Light and electron microscopic characteristics of the various lung endocrine cell types. *Invest Cell Pathol*, **3**, 345–51.

Haies, D. M., Gil, J. & Weibel, E. R. (1981) Morphometric study of rat lung cells. I. Numerical and dimensional characteristics of parenchymal cell population. *Am Rev Respir Dis*, **123**, 533–41.

Hajj, R., Baranek, T., Le Naour, R., Lesimple, P., Puchelle, E. & Coraux, C. (2007) Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties. *Stem Cells*, **25**, 139–48.

Hallman, M. & Teramo, K. (1981) Measurement of the lecithin/sphingomyelin ratio and phosphatidylglycerol in aminotic fluid: an accurate method for the assessment of fetal lung maturity. *Br J Obstet Gynaecol*, **88**, 806–13.

Hashimoto, N., Jin, H., Liu, T., Chensue, S. W. & Phan, S. H. (2004) Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest*, **113**, 243–52.

Herzog, E. L., Chai, L. & Krause, D. S. (2003) Plasticity of marrow-derived stem cells. *Blood*, **102**, 3483–93.

Holt, P. G., Schon-Hegrad, M. A. & McMenamin, P. G. (1990) Dendritic cells in the respiratory tract. *Int Rev Immunol*, **6**, 139–49.

Holtzman, M. J., Aizawa, H., Nadel, J. A. & Goetzl, E. J. (1983) Selective generation of leukotriene B4 by tracheal epithelial cells from dogs. *Biochem Biophys Res Commun*, **114**, 1071–6.

Hong, K. U., Reynolds, S. D., Giangreco, A., Hurley, C. M. & Stripp, B. R. (2001) Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am J Respir Cell Mol Biol*, **24**, 671–81.

Hong, K. U., Reynolds, S. D., Watkins, S., Fuchs, E. & Stripp, B. R. (2004a) Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol*, **164**, 577–88.

Hong, K. U., Reynolds, S. D., Watkins, S., Fuchs, E. & Stripp, B. R. (2004b) In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *Am J Physiol Lung Cell Mol Physiol*, **286**, L643–9.

Hoyt, R. F., Jr., Feldman, H. & Sorokin, S. P. (1982a) Neuroepithelial bodies (NEB) and solitary endocrine cells in the hamster lung. *Exp Lung Res*, **3**, 299–311.

Hoyt, R. F., Jr., Sorokin, S. P. & Feldman, H. (1982b) Small-granule (neuro)endocrine cells in the infracardiac lobe of a hamster lung. Number, subtypes, and distribution. *Exp Lung Res*, **3**, 273–98.

Inayama, Y., Hook, G. E., Brody, A. R., Cameron, G. S., Jetten, A. M., Gilmore, L. B., Gray, T. & Nettesheim, P. (1988) The differentiation potential of tracheal basal cells. *Lab Invest*, **58**, 706–17.

Inayama, Y., Hook, G. E., Brody, A. R., Jetten, A. M., Gray, T., Mahler, J. & Nettesheim, P. (1989) In vitro and in vivo growth and differentiation of clones of tracheal basal cells. *Am J Pathol*, **134**, 539–49.

Inglis, S. K., Corboz, M. R., Taylor, A. E. & Ballard, S. T. (1997) In situ visualization of bronchial submucosal glands and their secretory response to acetylcholine. *Am J Physiol*, **272**, L203–10.

Ishii, G., Sangai, T., Sugiyama, K., Ito, T., Hasebe, T., Endoh, Y., Magae, J. & Ochiai, A. (2005) In vivo characterization of bone marrow-derived fibroblasts recruited into fibrotic lesions. *Stem Cells*, **23**, 699–706.

Ishizawa, K., Kubo, H., Yamada, M., Kobayashi, S., Numasaki, M., Ueda, S., Suzuki, T. & Sasaki, H. (2004) Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema. *FEBS Lett*, **556**, 249–52.

Ito, T., Ikemi, Y., Ohmori, K., Kitamura, H. & Kanisawa, M. (1994) Airway epithelial cell changes in rats exposed to 0.25 ppm ozone for 20 months. *Exp Toxicol Pathol*, **46**, 1–6.

Jeffery, P. K. (1983) Morphologic features of airway surface epithelial cells and glands. *Am Rev Respir Dis*, **128**, S14–20.

Jeffery, P. K. (1987) The origins of secretions in the lower respiratory tract. *Eur J Respir Dis Suppl*, **153**, 34–42.

Jeffery, P. K. & Reid, L. (1975) New observations of rat airway epithelium: a quantitative and electron microscopic study. *J Anat*, **120**, 295–320.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A. & Verfaillie, C. M. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*, **418**, 41–9.

Johnson, D. E., Lock, J. E., Elde, R. P. & Thompson, T. R. (1982) Pulmonary neuroendocrine cells in hyaline membrane disease and bronchopulmonary dysplasia. *Pediatr Res*, **16**, 446–54.

Johnson, N. F. & Hubbs, A. F. (1990) Epithelial progenitor cells in the rat trachea. *Am J Respir Cell Mol Biol*, **3**, 579–85.

Johnson, N. F., Wilson, J. S., Habbersett, R., Thomassen, D. G., Shopp, G. M. & Smith, D. M. (1990) Separation and characterization of basal and secretory cells from the rat trachea by flow cytometry. *Cytometry*, **11**, 395–405.

Jones, A. W. & Reeve, N. L. (1978) Ultrastructural study of bleomycin-induced pulmonary changes in mice. *J Pathol*, **124**, 227–33.

Joo, N. S., Irokawa, T., Wu, J. V., Robbins, R. C., Whyte, R. I. & Wine, J. J. (2002) Absent secretion to vasoactive intestinal peptide in cystic fibrosis airway glands. *J Biol Chem*, **277**, 50710–5.

Kapanci, Y., Weibel, E. R., Kaplan, H. P. & Robinson, F. R. (1969) Pathogenesis and reversibility of the pulmonary lesions of oxygen toxicity in monkeys. II. Ultrastructural and morphometric studies. *Lab Invest*, **20**, 101–18.

Kikkawa, Y. & Yoneda, K. (1974) The type II epithelial cell of the lung. I. Method of isolation. *Lab Invest*, **30**, 76–84.

Kim, C. F., Jackson, E. L., Woolfenden, A. E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R. T. & Jacks, T. (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*, **121**, 823–35.

Kleeberger, W., Versmold, A., Rothamel, T., Glockner, S., Bredt, M., Haverich, A., Lehmann, U. & Kreipe, H. (2003) Increased chimerism of bronchial and alveolar epithelium in human lung allografts undergoing chronic injury. *Am J Pathol*, **162**, 1487–94.

Knowles, M. R. & Boucher, R. C. (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest*, **109**, 571–7.

Korbling, M. & Estrov, Z. (2003) Adult stem cells for tissue repair – a new therapeutic concept? *N Engl J Med*, **349**, 570–82.

Kotton, D. N., Ma, B. Y., Cardoso, W. V., Sanderson, E. A., Summer, R. S., Williams, M. C. & Fine, A. (2001) Bone marrow-derived cells as progenitors of lung alveolar epithelium. *Development*, **128**, 5181–8.

Kotton, D. N., Fabian, A. J. & Mulligan, R. C. (2005) Failure of bone marrow to reconstitute lung epithelium. *Am J Respir Cell Mol Biol*, **33**, 328–34.

Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. & Sharkis, S. J. (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*, **105**, 369–77.

Kuperman, D. A., Huang, X., Koth, L. L., Chang, G. H., Dolganov, G. M., Zhu, Z., Elias, J. A., Sheppard, D. & Erle, D. J. (2002) Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med*, **8**, 885–9.

Lamb, D. & Reid, L. (1969) Histochemical types of acidic glycoprotein produced by mucous cells of the tracheobronchial glands in man. *J Pathol*, **98**, 213–29.

Lauweryns, J. M. & Cokelaere, M. (1973) Hypoxia-sensitive neuro-epithelial bodies. Intrapulmonary secretory neuroreceptors, modulated by the CNS. *Z Zellforsch Mikrosk Anat*, **145**, 521–40.

Lauweryns, J. M., De Bock, V., Guelinckx, P. & Decramer, M. (1983) Effects of unilateral hypoxia on neuroepithelial bodies in rabbit lungs. *J Appl Physiol*, **55**, 1665–8.

Lawson, G. W., Van Winkle, L. S., Toskala, E., Senior, R. M., Parks, W. C. & Plopper, C. G. (2002) Mouse strain modulates the role of the ciliated cell in acute tracheobronchial airway injury-distal airways. *Am J Pathol*, **160**, 315–27.

Li, K., Nagalla, S. R. & Spindel, E. R. (1994) A rhesus monkey model to characterize the role of gastrin-releasing peptide (GRP) in lung development. Evidence for stimulation of airway growth. *J Clin Invest*, **94**, 1605–15.

Liu, X., Driskell, R. R. & Engelhardt, J. F. (2006) Stem cells in the lung. *Methods Enzymol*, **419**, 285–321.

Loi, R., Beckett, T., Goncz, K. K., Suratt, B. T. & Weiss, D. J. (2006) Limited restoration of cystic fibrosis lung epithelium in vivo with adult bone marrow-derived cells. *Am J Respir Crit Care Med*, **173**, 171–9.

Low, F. N. (1952) Electron microscopy of the rat lung. *Anat Rec*, **113**, 437–49.

Luciano, L., Reale, E. & Ruska, H. (1968) [On a 'chemoreceptive' sensory cell in the trachea of the rat]. *Z Zellforsch Mikrosk Anat*, **85**, 350–75.

Lumsden, A. B., McLean, A. & Lamb, D. (1984) Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax*, **39**, 844–9.

Macklin, C. C. (1954) The pulmonary alveolar mucoid film and the pneumonocytes. *Lancet*, **266**, 1099–1104.

Macpherson, H., Keir, P., Webb, S., Samuel, K., Boyle, S., Bickmore, W., Forrester, L. & Dorin, J. (2005) Bone marrow-derived SP cells can contribute to the respiratory tract of mice in vivo. *J Cell Sci*, **118**, 2441–50.

Magno, M. G. & Fishman, A. P. (1982) Origin, distribution, and blood flow of bronchial circulation in anesthetized sheep. *J Appl Physiol*, **53**, 272–9.

Mariassy, A. T., Plopper, C. G., St George, J. A. & Wilson, D. W. (1988a) Tracheobronchial epithelium of the sheep: IV. Lectin histochemical characterization of secretory epithelial cells. *Anat Rec*, **222**, 49–59.

Mariassy, A. T., St George, J. A., Nishio, S. J. & Plopper, C. G. (1988b) Tracheobronchial epithelium of the sheep: III. Carbohydrate histochemical and cytochemical characterization of secretory epithelial cells. *Anat Rec*, **221**, 540–9.

Mason, R. J., Williams, M. C., Moses, H. L., Mohla, S. & Berberich, M. A. (1997) Stem cells in lung development, disease, and therapy. *Am J Respir Cell Mol Biol*, **16**, 355–63.

Mattsson, J., Jansson, M., Wernerson, A. & Hassan, M. (2004) Lung epithelial cells and type II pneumocytes of donor origin after allogeneic hematopoietic stem cell transplantation. *Transplantation*, **78**, 154–7.

Matulionis, D. H. (1972) Analysis of the developing avian glycogen body. I. Ultrastructural morphology. *J Morphol*, **137**, 463–81.

Meyrick, B. & Reid, L. (1968) The alveolar brush cell in rat lung – a third pneumonocyte. *J Ultrastruct Res*, **23**, 71–80.

Meyrick, B. & Reid, L. (1970) Ultrastructure of cells in the human bronchial submucosal glands. *J Anat*, **107**, 281–99.

Meyrick, B., Sturgess, J. M. & Reid, L. (1969) A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. *Thorax*, **24**, 729–36.

Moore, B. B., Kolodsick, J. E., Thannickal, V. J., Cooke, K., Moore, T. A., Hogaboam, C., Wilke, C. A. & Toews, G. B. (2005) CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol*, **166**, 675–84.

Nettesheim, P., Jetten, A. M., Inayama, Y., Brody, A. R., George, M. A., Gilmore, L. B., Gray, T. & Hook, G. E. (1990) Pathways of differentiation of airway epithelial cells. *Environ Health Perspect*, **85**, 317–29.

Neuringer, I. P. & Randell, S. H. (2004) Stem cells and repair of lung injuries. *Respir Res*, **5**, 6.

Nishimura, D. Y., Fath, M., Mullins, R. F., Searby, C., Andrews, M., Davis, R., Andorf, J. L., Mykytyn, K., Swiderski, R. E., Yang, B., Carmi, R., Stone, E. M. & Sheffield, V. C. (2004) Bbs2-null mice have neurosensory deficits, a defect in social dominance, and retinopathy associated with mislocalization of rhodopsin. *Proc Natl Acad Sci U S A*, **101**, 16588–93.

Nishimura, Y., Hamazaki, T. S., Komazaki, S., Kamimura, S., Okochi, H. & Asashima, M. (2006) Ciliated cells differentiated from mouse embryonic stem cells. *Stem Cells*, **24**, 1381–8.

Ortiz, L. A., Gambelli, F., McBride, C., Gaupp, D., Baddoo, M., Kaminski, N. & Phinney, D. G. (2003) Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A*, **100**, 8407–11.

Paine, R., 3rd & Simon, R. H. (1996) Expanding the frontiers of lung biology through the creative use of alveolar epithelial cells in culture. *Am J Physiol*, **270**, L484–6.

Paine, R., Ben-Ze'ev, A., Farmer, S. R. & Brody, J. S. (1988) The pattern of cytokeratin synthesis is a marker of type 2 cell differentiation in adult and maturing fetal lung alveolar cells. *Dev Biol*, **129**, 505–15.

Pan, J., Yeger, H. & Cutz, E. (2002) Neuronal developmental marker FORSE-1 identifies a putative progenitor of the pulmonary neuroendocrine cell lineage during lung development. *J Histochem Cytochem*, **50**, 1567–78.

Park, J., Fang, S. & Adler, K. B. (2006a) Regulation of airway mucin secretion by MARCKS protein involves the chaperones heat shock protein 70 and cysteine string protein. *Proc Am Thorac Soc*, **3**, 493.

Park, K. S., Wells, J. M., Zorn, A. M., Wert, S. E., Laubach, V. E., Fernandez, L. G. & Whitsett, J. A. (2006b) Transdifferentiation of ciliated cells during repair of the respiratory epithelium. *Am J Respir Cell Mol Biol*, **34**, 151–7.

Pereira, R. F., Halford, K. W., O'Hara, M. D., Leeper, D. B., Sokolov, B. P., Pollard, M. D., Bagasra, O. & Prockop, D. J. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc Natl Acad Sci U S A*, **92**, 4857–61.

Phelps, D. S. & Floros, J. (1991) Localization of pulmonary surfactant proteins using immunohistochemistry and tissue in situ hybridization. *Exp Lung Res*, **17**, 985–95.

Phillips, R. J., Burdick, M. D., Hong, K., Lutz, M. A., Murray, L. A., Xue, Y. Y., Belperio, J. A., Keane, M. P. & Strieter, R. M. (2004) Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest*, **114**, 438–46.

Plopper, C. G., Dungworth, D. L. & Tyler, W. S. (1973) Ultrastructure of pulmonary alveolar macrophages in situ in lungs from rats exposed to ozone. *Am Rev Respir Dis*, **108**, 632–8.

Plopper, C. G., Hill, L. H. & Mariassy, A. T. (1980a) Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung. III. A study of man with comparison of 15 mammalian species. *Exp Lung Res*, **1**, 171–80.

Plopper, C. G., Mariassy, A. T. & Hill, L. H. (1980b) Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung: I. A comparison of rabbit, guinea pig, rat, hamster, and mouse. *Exp Lung Res*, **1**, 139–54.

Plopper, C. G., Mariassy, A. T. & Hill, L. H. (1980c) Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung: II. A comparison of horse, steer, sheep, dog, and cat. *Exp Lung Res*, **1**, 155–69.

Plopper, C. G., Alley, J. L., Serabjitsingh, C. J. & Philpot, R. M. (1983a) Cytodifferentiation of the nonciliated bronchiolar epithelial (Clara) cell during rabbit lung maturation: an ultrastructural and morphometric study. *Am J Anat*, **167**, 329–57.

Plopper, C. G., Halsebo, J. E., Berger, W. J., Sonstegard, K. S. & Nettesheim, P. (1983b) Distribution of nonciliated bronchiolar epithelial (Clara) cells in intra- and extrapulmonary airways of the rabbit. *Exp Lung Res*, **5**, 79–98.

Plopper, C. G., Mariassy, A. T. & Lollini, L. O. (1983c) Structure as revealed by airway dissection. A comparison of mammalian lungs. *Am Rev Respir Dis*, **128**, S4–7.

Plopper, C. G., Mariassy, A. T., Wilson, D. W., Alley, J. L., Nishio, S. J. & Nettesheim, P. (1983d) Comparison of nonciliated tracheal epithelial cells in six mammalian species: ultrastructure and population densities. *Exp Lung Res*, **5**, 281–94.

Plopper, C. G., Weir, A. J., Nishio, S. J., Cranz, D. L. & St George, J. A. (1986) Tracheal submucosal gland development in the rhesus monkey, *Macaca mulatta*: ultrastructure and histochemistry. *Anat Embryol (Berl)*, **174**, 167–78.

Plopper, C. G., Cranz, D. L., Kemp, L., Serabjit-Singh, C. J. & Philpot, R. M. (1987) Immunohistochemical demonstration of cytochrome P-450 monooxygenase in Clara cells throughout the tracheobronchial airways of the rabbit. *Exp Lung Res*, **13**, 59–68.

Plopper, C. G., Heidsiek, J. G., Weir, A. J., George, J. A. & Hyde, D. M. (1989) Tracheobronchial epithelium in the adult rhesus monkey: a quantitative histochemical and ultrastructural study. *Am J Anat*, **184**, 31–40.

Prockop, D. J. (2003) Further proof of the plasticity of adult stem cells and their role in tissue repair. *J Cell Biol*, **160**, 807–9.

Puchelle, E. & Peault, B. (2000) Human airway xenograft models of epithelial cell regeneration. *Respir Res*, **1**, 125–8.

Reddy, R., Buckley, S., Doerken, M., Barsky, L., Weinberg, K., Anderson, K. D., Warburton, D. & Driscoll, B. (2004) Isolation of a putative progenitor subpopulation of alveolar epithelial type 2 cells. *Am J Physiol Lung Cell Mol Physiol*, **286**, L658–67.

Reynolds, S. D., Giangreco, A., Power, J. H. & Stripp, B. R. (2000a) Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am J Pathol*, **156**, 269–78.

Reynolds, S. D., Hong, K. U., Giangreco, A., Mango, G. W., Guron, C., Morimoto, Y. & Stripp, B. R. (2000b) Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. *Am J Physiol Lung Cell Mol Physiol*, **278**, L1256–63.

Reynolds, S. D., Giangreco, A., Hong, K. U., McGrath, K. E., Ortiz, L. A. & Stripp, B. R. (2004) Airway injury in lung disease pathophysiology: selective depletion of airway stem and progenitor cell pools potentiates lung inflammation and alveolar dysfunction. *Am J Physiol Lung Cell Mol Physiol*, **287**, L1256–65.

Rhodin, J. A. (1966) The ciliated cell. Ultrastructure and function of the human tracheal mucosa. *Am Rev Respir Dis*, **93**, Suppl:1–15.

Rhodin, J. & Dalhamn, T. (1956) Electron microscopy of the tracheal ciliated mucosa in rat. *Z Zellforsch Mikrosk Anat*, **44**, 345–412.

Rippon, H. J., Ali, N. N., Polak, J. M. & Bishop, A. E. (2004) Initial observations on the effect of medium composition on the differentiation of murine embryonic stem cells to alveolar type II cells. *Cloning Stem Cells*, **6**, 49–56.

Rippon, H. J., Polak, J. M., Qin, M. & Bishop, A. E. (2006) Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. *Stem Cells*, **24**, 1389–98.

Rogers, D. F. (2000) Mucus pathophysiology in COPD: differences to asthma, and pharmacotherapy. *Monaldi Arch Chest Dis*, **55**, 324–32.

Rogers, D. F. (2004) Airway mucus hypersecretion in asthma: an undervalued pathology? *Curr Opin Pharmacol*, **4**, 241–50.

Rojas, M., Xu, J., Woods, C. R., Mora, A. L., Spears, W., Roman, J. & Brigham, K. L. (2005) Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol*, **33**, 145–52.

Rooney, S. A., Young, S. L. & Mendelson, C. R. (1994) Molecular and cellular processing of lung surfactant. *Faseb J*, **8**, 957–67.

Rose, M. C., Nickola, T. J. & Voynow, J. A. (2001) Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. *Am J Respir Cell Mol Biol*, **25**, 533–7.

Ryan, U. S., Ryan, J. W. & Smith, D. S. (1975) Alveolar type II cells: studies on the mode of release of lamellar bodies. *Tissue Cell*, **7**, 587–99.

Salinas, D., Haggie, P. M., Thiagarajah, J. R., Song, Y., Rosbe, K., Finkbeiner, W. E., Nielson, D. W. & Verkman, A. S. (2005) Submucosal gland dysfunction as a primary defect in cystic fibrosis. *Faseb J*, **19**, 431–3.

Samadikuchaksaraei, A., Cohen, S., Isaac, K., Rippon, H. J., Polak, J. M., Bielby, R. C. & Bishop, A. E. (2006) Derivation of distal airway epithelium from human embryonic stem cells. *Tissue Eng*, **12**, 867–75.

Satir, P. (1999) The cilium as a biological nanomachine. *Faseb J*, **13** Suppl 2. S235–7.

Scheuermann, D. W. (1987) Morphology and cytochemistry of the endocrine epithelial system in the lung. *Int Rev Cytol*, **106**, 35–88.

Schmidt, M., Sun, G., Stacey, M. A., Mori, L. & Mattoli, S. (2003) Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol*, **171**, 380–9.

Schneeberger, E. E. & Hamelin, M. (1984) Interaction of serum proteins with lung endothelial glycocalyx: its effect on endothelial permeability. *Am J Physiol*, **247**, H206–17.

Schoeberlein, A., Holzgreve, W., Dudler, L., Hahn, S. & Surbek, D. V. (2005) Tissue-specific engraftment after in utero transplantation of allogeneic mesenchymal stem cells into sheep fetuses. *Am J Obstet Gynecol*, **192**, 1044–52.

Serafini, S. M. & Michaelson, E. D. (1977) Length and distribution of cilia in human and canine airways. *Bull Eur Physiopathol Respir*, **13**, 551–9.

Shannon, J. M., Jennings, S. D. & Nielsen, L. D. (1992) Modulation of alveolar type II cell differentiated function in vitro. *Am J Physiol*, **262**, L427–36.

Shimizu, T., Nishihara, M., Kawaguchi, S. & Sakakura, Y. (1994) Expression of phenotypic markers during regeneration of rat tracheal epithelium following mechanical injury. *Am J Respir Cell Mol Biol*, **11**, 85–94.

Simionescu, D. & Simionescu, M. (1983) Differentiated distribution of the cell surface charge on the alveolar-capillary unit. Characteristic paucity of anionic sites on the air-blood barrier. *Microvasc Res*, **25**, 85–100.

Singer, M., Martin, L. D., Vargaftig, B. B., Park, J., Gruber, A. D., Li, Y. & Adler, K. B. (2004) A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. *Nat Med*, **10**, 193–6.

Singh, G., Katyal, S. L., Brown, W. E., Kennedy, A. L., Singh, U. & Wong-Chong, M. L. (1990) Clara cell 10 kDa protein (CC10): comparison of structure and function to uteroglobin. *Biochim Biophys Acta*, **1039**, 348–55.

Sirois, P. & Cadieux, A. (1986) Converting enzyme activity of free airway cells. *Inflammation*, **10**, 293–302.

Sleigh, M. A., Blake, J. R. & Liron, N. (1988) The propulsion of mucus by cilia. *Am Rev Respir Dis*, **137**, 726–41.

Sorokin, S. P. & Hoyt, R. F., Jr. (1982) Development of neuroepithelial bodies and solitary endocrine cells in fetal rabbit lungs. II. Nonspecific esterase as an indicator of early maturation. *Exp Lung Res*, **3**, 261–72.

Sorokin, S. P., Hoyt, R. F., Jr. & McNelly, N. A. (1989) Nonimmune-mediated phagocytosis by 'premedullary' lung macrophages: effects of concanavalin A, tuftsin, and macrophage-inhibitory peptide. *Anat Rec*, **223**, 55–61.

Spencer, H., Rampling, D., Aurora, P., Bonnet, D., Hart, S. L. & Jaffe, A. (2005) Transbronchial biopsies provide longitudinal evidence for epithelial chimerism in children following sex mismatched lung transplantation. *Thorax*, **60**, 60–2.

Spicer, S. S., Chakrin, L. W., Wardell, J. R., Jr. & Kendrick, W. (1971) Histochemistry of mucosubstances in the canine and human respiratory tract. *Lab Invest*, **25**, 483–90.

Stevens, T. P., McBride, J. T., Peake, J. L., Pinkerton, K. E. & Stripp, B. R. (1997) Cell proliferation contributes to PNEC hyperplasia after acute airway injury. *Am J Physiol*, **272**, L486–93.

Sunday, M. E. & Willett, C. G. (1992) Induction and spontaneous regression of pulmonary neuroendocrine cell hyperplasia in a hamster model. *Chest*, **101**, 21S.

Sunday, M. E., Kaplan, L. M., Motoyama, E., Chin, W. W. & Spindel, E. R. (1988) Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab Invest*, **59**, 5–24.

Suratt, B. T., Cool, C. D., Serls, A. E., Chen, L., Varella-Garcia, M., Shpall, E. J., Brown, K. K. & Worthen, G. S. (2003) Human pulmonary chimerism after hematopoietic stem cell transplantation. *Am J Respir Crit Care Med*, **168**, 318–22.

Tateishi, R. (1973) Distribution of argyrophil cells in adult human lungs. *Arch Pathol*, **96**, 198–202.

Temann, U. A., Prasad, B., Gallup, M. W., Basbaum, C., Ho, S. B., Flavell, R. A. & Rankin, J. A. (1997) A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion. *Am J Respir Cell Mol Biol*, **16**, 471–8.

Theise, N. D., Henegariu, O., Grove, J., Jagirdar, J., Kao, P. N., Crawford, J. M., Badve, S., Saxena, R. & Krause, D. S. (2002) Radiation pneumonitis in mice: a severe injury model for pneumocyte engraftment from bone marrow. *Exp Hematol*, **30**, 1333–8.

Tyler, W. S. (1983) Comparative subgross anatomy of lungs. Pleuras, interlobular septa, and distal airways. *Am Rev Respir Dis*, **128**, S32–6.

Tyner, J. W., Kim, E. Y., Ide, K., Pelletier, M. R., Roswit, W. T., Morton, J. D., Battaile, J. T., Patel, A. C., Patterson, G. A., Castro, M., Spoor, M. S., You, Y., Brody, S. L. & Holtzman, M. J. (2006) Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. *J Clin Invest*, **116**, 309–21.

Uhal, B. D. (1997) Cell cycle kinetics in the alveolar epithelium. *Am J Physiol*, **272**, L1031–45.
Vassilopoulos, G., Wang, P. R. & Russell, D. W. (2003) Transplanted bone marrow regenerates liver by cell fusion. *Nature*, **422**, 901–4.
Vestbo, J., Prescott, E. & Lange, P. (1996) Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am J Respir Crit Care Med*, **153**, 1530–5.
Vogel, G. (1998) Interleukin-13's key role in asthma shown. *Science*, **282**, 2168.
Wagers, A. J., Christensen, J. L. & Weissman, I. L. (2002) Cell fate determination from stem cells. *Gene Ther*, **9**, 606–12.
Wang, D., Haviland, D. L., Burns, A. R., Zsigmond, E. & Wetsel, R. A. (2007) A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A*, **104**, 4449–54.
Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S. & Grompe, M. (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature*, **422**, 897–901.
Warburton, D., Bellusci, S., De Langhe, S., Del Moral, P. M., Fleury, V., Mailleux, A., Tefft, D., Unbekandt, M., Wang, K. & Shi, W. (2005) Molecular mechanisms of early lung specification and branching morphogenesis. *Pediatr Res*, **57**, 26R–37R.
Watson, J. H. & Brinkman, G. L. (1964) Electron microscopy of the epithelial cells of normal and bronchitic human bronchus. *Am Rev Respir Dis*, **90**, 851–66.
Weiss, D. J., Berberich, M. A., Borok, Z., Gail, D. B., Kolls, J. K., Penland, C. & Prockop, D. J. (2006) Adult stem cells, lung biology, and lung disease. NHLBI/Cystic Fibrosis Foundation Workshop. *Proc Am Thorac Soc*, **3**, 193–207.
Wharton, J., Polak, J. M., Bloom, S. R., Ghatei, M. A., Solcia, E., Brown, M. R. & Pearse, A. G. (1978) Bombesin-like immunoreactivity in the lung. *Nature*, **273**, 769–70.
Whittaker, L., Niu, N., Temann, U. A., Stoddard, A., Flavell, R. A., Ray, A., Homer, R. J. & Cohn, L. (2002) Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9. *Am J Respir Cell Mol Biol*, **27**, 593–602.
Widdicombe, J. G. (1987a) Role of lipids in airway function. *Eur J Respir Dis Suppl*, **153**, 197–204.
Widdicombe, J. H. (1987b) Fluid transport by airway epithelia. *Biorheology*, **24**, 551–5.
Widdicombe, J. G. & Pack, R. J. (1982) The Clara cell. *Eur J Respir Dis*, **63**, 202–20.
Widdicombe, J. H., Chen, L. L., Sporer, H., Choi, H. K., Pecson, I. S. & Bastacky, S. J. (2001) Distribution of tracheal and laryngeal mucous glands in some rodents and the rabbit. *J Anat*, **198**, 207–21.
Williams, M. C. (1984) Endocytosis in alveolar type II cells: effect of charge and size of tracers. *Proc Natl Acad Sci U S A*, **81**, 6054–8.
Williams, M. C. & Mason, R. J. (1977) Development of the type II cell in the fetal rat lung. *Am Rev Respir Dis*, **115**, 37–47.
Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T. Y., Karp, C. L. & Donaldson, D. D. (1998) Interleukin-13: central mediator of allergic asthma. *Science*, **282**, 2258–61.
Wright, J. R. & Dobbs, L. G. (1991) Regulation of pulmonary surfactant secretion and clearance. *Annu Rev Physiol*, **53**, 395–414.
Wright, J. R., Wager, R. E., Hamilton, R. L., Huang, M. & Clements, J. A. (1986) Uptake of lung surfactant subfractions into lamellar bodies of adult rabbit lungs. *J Appl Physiol*, **60**, 817–25.
Yamada, M., Kubo, H., Kobayashi, S., Ishizawa, K., Numasaki, M., Ueda, S., Suzuki, T. & Sasaki, H. (2004) Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. *J Immunol*, **172**, 1266–72.
Yamada, M., Kubo, H., Ishizawa, K., Kobayashi, S., Shinkawa, M. & Sasaki, H. (2005) Increased circulating endothelial progenitor cells in patients with bacterial pneumonia: evidence that bone marrow derived cells contribute to lung repair. *Thorax*, **60**, 410–3.
Yamaya, M., Finkbeiner, W. E. & Widdicombe, J. H. (1991) Ion transport by cultures of human tracheobronchial submucosal glands. *Am J Physiol*, **261**, L485–90.

Yoneda, K. & Walzer, P. D. (1984) The effect of corticosteroid treatment on the cell surface glycocalyx of the rat pulmonary alveolus: relevance to the host – parasite relationship in *Pneumocystis carinii* infection. *Br J Exp Pathol*, **65**, 347–54.

Zander, D. S., Baz, M. A., Cogle, C. R., Visner, G. A., Theise, N. D. & Crawford, J. M. (2005) Bone marrow-derived stem-cell repopulation contributes minimally to the Type II pneumocyte pool in transplanted human lungs. *Transplantation*, **80**, 206–12.

Zepeda, M. L., Chinoy, M. R. & Wilson, J. M. (1995) Characterization of stem cells in human airway capable of reconstituting a fully differentiated bronchial epithelium. *Somat Cell Mol Genet*, **21**, 61–73.

2

Epithelial Adhesive Structures and Adhesion Molecule Expression

George Su[1] and **Dean Sheppard**[2]
[1] *Department of Medicine, Division of Pulmonary and Critical Care, University of California, San Francisco, California, USA*
[2] *Lung Biology Center, University of California, San Francisco, California, USA*

2.1 Introduction

The respiratory tract is lined by a continuous layer of epithelial cells. Pseudostratified columnar epithelium made up of ciliated, goblet, and basal cells line the large proximal airway. In the distal airway, ciliated cells transition to a more cuboidal morphology and decrease in number, while basal cells and Clara cells become more prominent. Alveoli are lined by a thin monolayer of type I alveolar cells interspersed with type II alveolar cells. Together, these cells constitute a remarkable epithelial tissue that regulates host defence, inflammation, gas exchange, and barrier function. Barrier function and interactions with the surrounding microenvironment are determined by specialized structures residing at cell–cell and cell–substratum junctions. This chapter examines these structures, their relevance in the pulmonary epithelium, reviews their protein constituents and associations, and discusses newer insights into their functions and potential roles in disease.

2.2 Cell–cell adhesive structures

In 1870, the Italian scientist and microscopist Giulio Bizzozero designated the 'terminal bar', an apical 1–2 μm area of condensation observed at epithelial cell–cell junctions (Bizzozero, 1870). In 1963, Farquhar and Palade re-examined this structure using electron microscopy (EM) and discovered the tripartite 'junctional complex' (Farquhar and Palade, 1963). The three components, based on distinct morphologies and relative locations (Figure 2.1), were named the zonula occludens or 'closing belt', now referred to as the tight junction (TJ), the zonula adherens or 'adhering belt', now known as the adherens junction (AJ) or intermediate junction, and the macula adherens or 'adhering spot', now known as the desmosome. Analogous ultrastructure has been described in both non-mammalian and

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

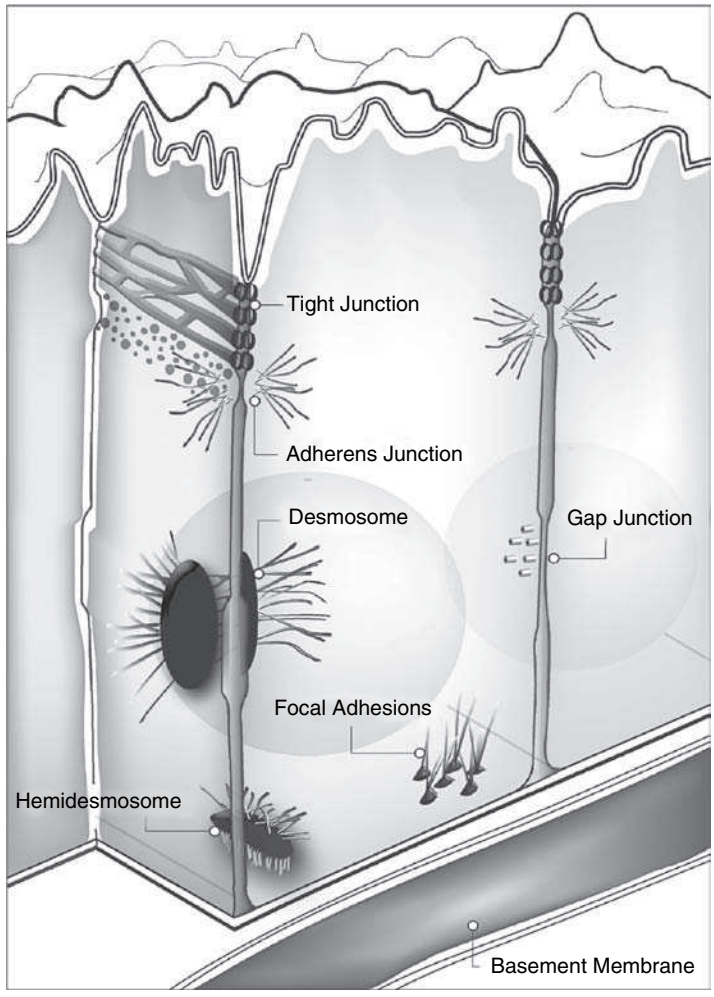


Figure 2.1 Epithelial cell–cell and cell–substratum adhesion structures: tight junctions, adherens junctions, desmosomes, hemidesmosomes, and focal adhesions. The TJ is the most apical member of the junctional complex. TJ strands and fibrils form a circumferential, gasket-like band. Below the TJ is the E-cadherin-rich AJ which is linked to intracellular actin through linker proteins to form another continuous band around the perimeter of the cell. Desmosomes form discrete disc-like adhesion sites which characteristically associate with intermediate filaments (IFs), rather than with actin. IFs loop from the desmosomal plaque to the cytoplasm, then back. Hemidesmosomes are specialized structures that mediate adhesion of the epithelial cell to the underlying basement membrane. Hemidesmosomes, like desmosomes, associate with IFs through its cytoplasmic plaque. Focal adhesions are regions of close apposition to the underlying extracellular matrix organized around links between integrins and the ends of actin filaments. Gap junctions permit intercellular metabolic coupling, but are not formally described as cell–cell, or cell–substratum adhesion structures

mammalian pulmonary epithelia. Gap junctions, or nexi, which permit metabolic coupling and direct transmission of small cytosolic signalling molecules between adjacent cells, are not considered part of the junctional complex and will not be specifically reviewed here.

2.2.1 Tight junctions

The TJ is the most apical member of the junctional complex (Figure 2.1). At low magnification, it forms a continuous intercellular belt-like zone, 0.1 to 0.7 μm in depth, where adjacent plasma membranes are juxtaposed. Higher magnification of this zone reveals punctate contacts where the outer lipid leaflets of conjoined plasma membranes merge, eliminating interposed extracellular space. These contacts correspond to the dramatic strands and fibrils seen in EM freeze-fracture replicas. Rat airway have TJ morphologies that vary between epithelial cell type (Schneeberger, 1980). In the trachea, TJs in ciliated cells have sparsely interconnected parallel luminal fibrils and large ablumenal fibril loops. In distal intrapulmonary airway, the luminal fibrils are highly interconnected. This TJ morphology is also seen with serous cells, epithelial 'brush cells', and Clara cells. In normal human bronchi, however, EM of these strand arrangements shows highly variable patterns from one junction to the next, irrespective of either airway distribution or cell type (Godfrey *et al*., 1992). In human alveolar epithelial cells, freeze-fracture replicas reveal a belt-like network of 3–7 superimposed fibrils that partition to the protoplasmic (P) face, with complementary grooves that partition to the exoplasmic (E) face (Bartels, 1979). It is generally assumed that these strands and fibrils represent polymers of interacting transmembrane proteins, although a contribution from lipids and specialized lipid structures cannot be ruled out (Tsukita *et al*., 2001). These transmembrane proteins terminate at a cytoplasmic plaque, originally described as a 0.2 to 0.5 μm 'diffuse band of dense cytoplasmic material' (Farquhar and Palade, 1963).

The first major advance in defining the molecular composition of TJs occurred in 1993, when the Tsukita group used an isolated junction-enriched fraction from chick liver as an antigen to generate monoclonal antibodies. Prior to this, attempts to raise antibodies recognizing the highly-conserved TJ structure in mammals were not successful. Both occludin (Furuse *et al*., 1993) and the first set of claudins (Furuse *et al*., 1998a) were discovered. Soon thereafter, a novel member of the immunoglobulin superfamily, termed the junctional adhesion molecule (JAM), became the third type of transmembrane protein known to exist in TJs (Martin-Padura *et al*., 1998).

A rapidly growing number of TJ constituents have since been identified, supporting the concept that the TJ is an elaborate multifunctional protein complex. TJs contain upwards of 40 different proteins, including products of multigene families, which are arranged with characteristic adhesion complex architecture, consisting of a set of transmembrane proteins, a large number of cytoplasmic adaptor proteins, and a group of miscellaneous proteins that interact either directly or indirectly with the cytoplasmic plaque.

Occludin

Occludin is a 60-kDa tetraspan protein that orients two extracellular domain loops, characteristically rich in glycine and tyrosine residues, between cytosolic amino (N)- and carboxy (C)-terminal domains (Figure 2.2). Human occludin is the product of a single gene located on chromosome band 5q13.1 (Saitou *et al*., 1997). Occludin mRNA has been shown to be highly expressed in the testis, kidney, liver, lung, and brain – all tissues that bear well-developed TJs (Saitou *et al*., 1997). Splice variants of occludin have been identified in human colonic epithelial cells (Mankertz *et al*., 2002), Madin–Darby canine kidney (MDCK) cells (Muresan *et al*., 2000), and in many mammalian tissues including the human bronchial epithelium (transmembrane domain 4-deficient isoform (TM4-)) (Ghassemifar *et al*., 2002).

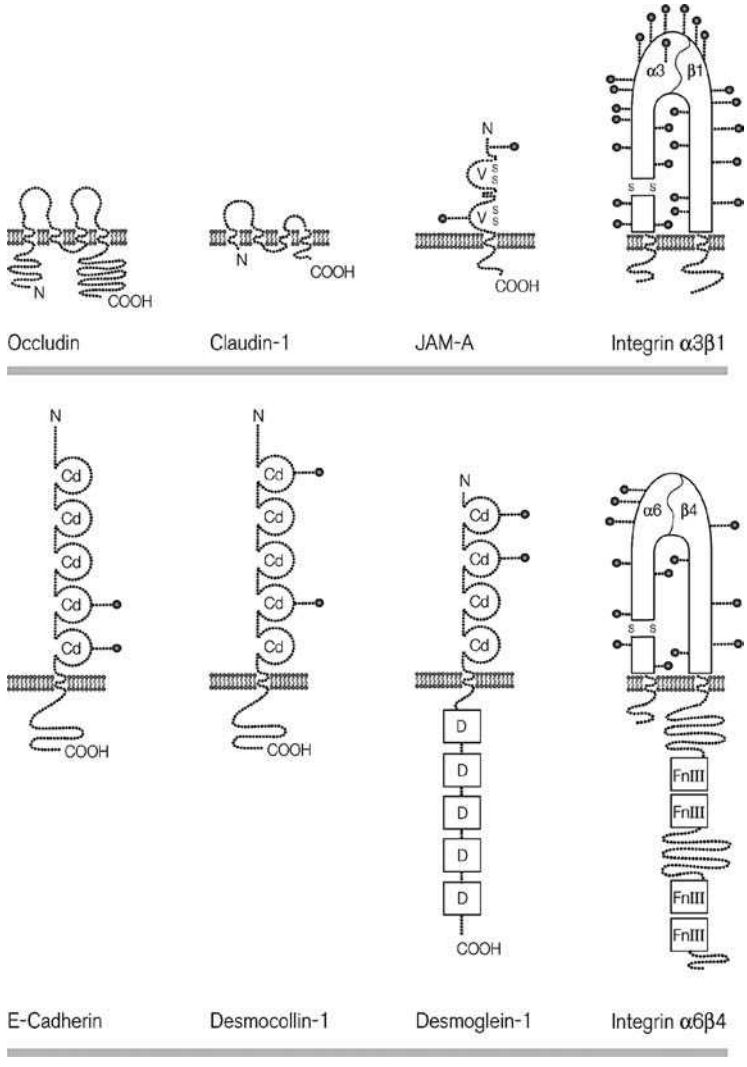


Figure 2.2 Epithelial transmembrane cell–cell and cell–substratum adhesion proteins. Tight junction: occludin, claudin-1, and junction adhesion molecule (JAM)-A. Occludin contains a first extracellular loop that is characteristically rich in tyrosine and glycine residues and a C-terminal PDZ domain. In contrast, the amino acid composition of the two extracellular loops of claudin varies significantly among different claudins. Claudins (except for claudin-12) contain a C-terminal PDZ motif (conserved YV sequence). JAM-A spans the plasma membrane once and has two extracellular Ig type domains (V), of which the first loop is required for homotypic binding between cells. Adherens junction: epithelial (E)-cadherin is a classical type I cadherin containing five cadherin repeats (Cd) of approximately 110 amino acids separated by four calcium binding sites. The N-terminus contains the conserved HAV motif required for homophilic binding. Desmosomal cadherens: desmocollin-1 and desmoglein-1. Desmocollin-1 is a classical type I cadherin that participates in homodimeric and homotypic binding. Its short conserved C-terminus interacts with intermediate filaments. Demoglein-1 differs from desmocollin-1 in that it contains a short propeptide and only four Cd repeats. Furthermore, its intracellular domain contains five, 28–30 amino acid-long repeat sequences (desmoglein repeats) (D)

Splice variants have been suggested to differentially affect TJ adhesion. In epithelial cells, occludin undergoes various post-translational modifications, including targeted phosphorylation (Stuart and Nigam, 1995), which is thought to affect occludin assembly at the TJ. Occludin is a target substrate for multiple kinases, including non-receptor tyrosine kinases Src and Yes (Chen *et al*., 2002; Kale *et al*., 2003), and serine/threonine kinases, such as casein kinase (CK)2 (Smales *et al*., 2003) and protein kinase C (PKC) (Andreeva *et al*., 2001).

Approximately half of the 522 amino acid residues of occludin are contained within its long cytoplasmic C-terminal tail (Ando-Akatsuka *et al*., 1996). The last 150 amino acids of this tail interact directly with F-actin (Wittchen *et al*., 1999). This property is unique to occludin, and not shared by other TJ integral proteins, which require protein adaptors. The cytoplasmic tail also interacts with a large number of proteins at the TJ plaque (Nusrat *et al*., 2000). These include scaffolding proteins such as cingulin (Citi *et al*., 1988) and zonula occludens (ZO)-1 (Fanning *et al*., 1998), ZO-2 (Itoh *et al*., 1999a), and ZO-3 (Wittchen *et al*., 2000), and the membrane trafficking protein VAMP (vesicle-associated membrane protein, or synaptobrevin)-associated protein of 33 kDa (VAP33) (Lapierre *et al*., 1999). ZO belongs to a family of multidomain scaffolding proteins known as membrane-associated guanylate kinase (MAGUK) homologues, all of which contain several binding domains (e.g. src homology (SH)3 and post-synaptic density protein-Drosophila disc large tumour suppressor-ZO-1 (PDZ) (Ranganathan and Ross, 1997; Lockless and Ranganathan, 1999)) and an enzymatically inactive guanylate kinase (GK) domain. The recently solved crystal structure of the occludin-ZO-1 binding site (Li *et al*., 2005) may provide additional insights into this highly-conserved interaction.

Initial experimental data suggested that occludin might be the principal protein required to maintain TJ structure and adhesive function (Furuse *et al*., 1996). However, examination of epithelia in mice deficient in occludin did not reveal obvious differences in epithelial barrier function (Saitou *et al*., 2000). Epithelial claudins, rather, are now considered the essential determinants of TJ structure and function. In retrospect, since occludin is a single gene product that lacks an extracellular charge, it seems unlikely that it could produce the kind of functional variety seen in TJs, including tissue-specific resistances and unique paracellular charge-specificities. On the other hand, occludin-deficient mice do display a complex phenotype that includes chronic inflammation and hyperplasia of the gastric epithelium, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of the salivary gland, and thinning of compact bone. Furthermore, occludin-deficient male mice are infertile (Saitou *et al*., 2000). This diverse phenotype may ultimately reflect complex occludin-mediated cell signalling, with each finding dependent on a specific cadre of plaque proteins. As of yet, occludin deficiency has not been associated with a phenotype specific to the lungs or to the pulmonary epithelium.

Figure 2.2 (Continued) The C-terminus expresses the adhesion motif, R/YAL. Integrins $\alpha3\beta1$ and $\alpha6\beta4$: $\alpha3\beta1$ contains an $\alpha3$ chain proteolytically cleaved into a heavy and a light chain, which are disulphide-bonded (S-S). $\alpha3\beta1$ has complex binding specificities to fibronectin, collagen, and laminin-5. $\alpha6\beta4$ is the primary integrin constituent of hemidesmosomes. Unique among integrin β chains, $\beta4$ contains a large 118 kDa cytoplasmic domain with four fibronectin type III repeats (FnIII). This cytoplasmic tail interacts with intermediate filaments, rather than with actin, as seen with other integrins. N-Glycosylation sites (grey circles), amine terminus (N), carboxyl terminus (COOH)

Claudins

In 1998, the first claudins, claudin-1 and -2, were co-purified with occludin (Furuse *et al*., 1998a). Database searching, and both cDNA and genomic cloning, have since expanded the claudin multigene family to include at least 24 members in humans and mice. Claudins are believed to be the essential determinants of both TJ structure and function. Claudin incorporates into TJ fibrils when expressed in MDCK cells, and forms TJ strands *de novo* when expressed in L fibroblasts lacking endogenous claudins (Furuse *et al*., 1998b). Furthermore, L-fibroblast claudin transfectants exhibit increased adhesion activity and form ultrastructural TJ 'kissing points' between adjacent cells (Kubota *et al*., 1999). L-fibroblasts expressing singlets or pairs of claudin-1, -2, and -3 produce TJ strands through homomeric and heteromeric claudin binding within individual strands, and homotypic and heterotypic binding between opposing strands (except between claudin-1 and -2) (Furuse *et al*., 1999).

Claudin genes encode 20–27 kDa proteins, none of which show sequence homology to occludin (Furuse *et al*., 1998a) (Figure 2.2). Like occludin, however, claudins are predicted to be tetraspan proteins with cytoplasmic N- and C-terminal domains (Furuse *et al*., 1998a). Claudins are recognized by a highly-conserved amino acid motif, GLWxxC-C, contained within the first extracellular loop (Van Itallie and Anderson, 2004). This first extracellular loop influences paracellular charge selectivity and resistance (Colegio *et al*., 2003). Diversity in this loop, outside of the conserved motif, may explain how claudins, or why a particular claudin repertoire, might determine paracellular ion specificity.

Despite having strikingly divergent C-terminal cytoplasmic domains, claudins all end (with the exception of claudin-12) in a PDZ-binding motif (most contain the conserved YV sequence). Claudins interact with the PDZ domains of a variety of proteins, including ZO-1, -2, and -3 (Itoh *et al*., 1999), the multi-PDZ domain protein (MUPP)-1 (Hamazaki *et al*., 2002), and the Protein Associated with Lin Seven (PALS)-1-associated TJ protein (PATJ) (Roh *et al*., 2002). ZO-1 and ZO-2 can independently determine whether and where claudins are polymerized (Umeda *et al*., 2006). PALS-1 and PATJ are thought to regulate apical-basal polarity in mammalian epithelial cells (Straight *et al*., 2004; Shin *et al*., 2005). Polarity is a fundamentally important feature of epithelial cells and epithelial cell function. Despite insights provided by predicted structure and known C-terminal binding partners, the functions associated with this domain remain unclear. L-fibroblasts expressing claudin mutants lacking almost all of the C-terminal cytoplasmic domain still form TJ strands (Furuse *et al*., 1999). Claudins lacking their last three amino acids, or those in which the PDZ-binding sites are blocked by epitope tagging, still localize to cell–cell contacts and form freeze-fracture strands (Furuse *et al*., 1998b). The strands formed by PDZ-blocked claudins, however, are poorly organized and not restricted to the apical border (McCarthy *et al*., 2000).

In the pulmonary epithelium, claudin expression varies with specific cell type and differentiation state, with changes in transepithelial permeability, and in response to transcription factors linked to lung branching morphogenesis. Transdifferentiation of rat alveolar epithelial type II cells to cells with a type I-like phenotype after prolonged culture or exposure to epidermal growth factor (EGF) is associated with increases in claudin-4 and -7 and decreases in claudin-3 and -5 expression (Chen *et al*., 2005). In adult rat lung sections, claudin-3, -4, and -5 are expressed in alveolar type II epithelial cells and claudin-5 is expressed throughout the alveolus (Wang *et al*., 2003). Induced increases in rat type II cell permeability

increases both claudin-3 and -5 expression (Wang *et al*., 2003). Claudin-1, -3, and -5 are expressed in normal airway of human lungs (Coyne *et al*., 2003). Heterologous expression of claudin-1 and -3 in IB3-1 human airway epithelial cells decreases solute permeability, while claudin-5 expression increases permeability (Coyne *et al*., 2003). Sphingosine 1-phosphate (S1P), a lipid mediator that induces pulmonary edema formation when administered through the airway, acts additively with tumour necrosis factor (TNF) to induce a rapid loss of claudin-18. These changes correlate with increased edema formation in a mouse model of acute lung injury (Gon *et al*., 2005). Claudin-18 is also uniquely down-regulated in T/ebp/Nkx2.1-deficient mouse embryo lungs (Niimi *et al*., 2001). The T/ebp/Nkx2.1 transcription factor is expressed in all pulmonary epithelial cells during early development and is considered an important regulator of pulmonary branching morphogenesis (Yuan *et al*., 2000).

Wide-ranging in vivo functions of claudins may be deduced from both human hereditary disorders caused by mutations of claudin genes and knockout mouse phenotypes. For example, mutation of a gene encoding claudin-14, which is expressed in the outer hair cells of the cochlea, is associated with profound autosomal recessive deafness (Wilcox *et al*., 2001). Claudin-16 (also known as paracellin-1) mutations cause familial hypomagnesemia with hypercalciuria and nephrocalcinosis (Simon *et al*., 1999). Consistent with this, the first extracellular loop of claudin-16 carries a characteristically negative charge which could determine divalent cation selectivity in the loop of Henle. Claudin-19-deficient mice walk awkwardly on smooth surfaces, a phenotype that has been attributed to a lack of TJs in myelin sheaths and subsequent defects in saltatory conduction in the peripheral nervous system (Miyamoto *et al*., 2005). Specific pulmonary diseases directly associated with claudin deficiency or specific claudin mutations have not yet been demonstrated. However, considering the diversity of the claudin family and heterogeneity of its attributed functions, one would expect that diseases involving, for example, pulmonary epithelial TJ regulation of paracellular permeability, sodium vectorial transport (Shlyonsky *et al*., 2005), and ion selectivity (e.g. acute lung injury, bronchitis, asthma, cystic fibrosis), epithelial cell differentiation (e.g. bronchogenic carcinoma), or aberrant lung development might reflect alterations in normal claudin function.

Junctional adhesion molecule

JAM-1 was the first protein belonging to the Ig superfamily identified at TJs (Martin-Padura *et al*., 1998). JAM-1 is now called JAM-A according to revised nomenclature (Muller, 2003).[1] JAM-A is a 43 kDa glycosylated protein characterized by two extracellular V-type Ig domains, a single transmembrane domain, and a short intracellular C-terminal domain containing a PDZ binding motif (Figure 2.2). JAM-A localizes to epithelial TJ strand-containing regions (Itoh *et al*., 2001) and forms homophilic contacts between V-type Ig domains of opposing JAMs. Although the subcellular localization of JAM-B and JAM-C has not been addressed by ultrastructural analysis, JAM-C has been shown to co-distribute

[1] The new nomenclature for JAMs applies identical names for mouse and human analogues. JAM-A corresponds to JAM, JAM-1, F11-receptor or the 106 antigen. JAM-B corresponds to mouse JAM-3 and human JAM-2 and vascular endothelial (VE)-JAM. JAM-C corresponds to mouse JAM-2 and to human JAM-3. Two JAM-like molecules JAM-4 and JAM-like (JAML) are more closely related to the coxsackie and adenovirus receptor (CAR) and the endothelial cell-selective adhesion molecule (ESAM).

with ZO-1 when expressed in polarized epithelial cells, suggesting that it, like JAM-A, is associated with epithelial TJs (Aurrand-Lions *et al*., 2001). In addition, human JAM-A is expressed in platelets and on circulating leukocytes including monocytes, neutrophils, and B- and T-lymphocytes (Williams *et al*., 1999); JAM-A facilitates migration of monocytes through the paracellular pathway (Martin-Padura *et al*., 1998; Del Maschio *et al*., 1999). JAM-B (also known as VE-JAM, mouse JAM-3, or human JAM-2) and JAM-C (also known as mouse JAM-2 or human JAM-3) are expressed in the endothelium of several different organs (Aurrand-Lions *et al*., 2001). Like JAM-A, JAM-C is expressed in platelets and by various human leukocyte subsets (Liang *et al*., 2002; Santoso *et al*., 2002). In human airway epithelium, JAM is expressed (Liu *et al*., 2000), but its specific function has yet to be determined.

Four additional Ig-superfamily members have been identified at TJs: JAM-4 (Hirabayashi *et al*., 2003), the coxsackie and adenovirus receptor (CAR) (Cohen *et al*., 2001), and CAR-like membrane protein (CLMP) (Raschperger *et al*., 2004) in epithelial cells, and endothelial cell-selective adhesion molecule (ESAM) (Nasdala *et al*., 2002) in endothelial cells. JAM-4 recruits ZO-1 and occludin to cell–cell contacts and mediates calcium-independent homophilic adhesion (Hirabayashi *et al*., 2003). CAR recruits ZO-1 to the cell membrane and, when overexpressed in epithelial cells, increases transepithelial resistance (Cohen *et al*., 2001). CLMP co-localizes with ZO-1 and occludin at TJs and also appears to regulate transepithelial resistance (Raschperger *et al*., 2004). While JAM-4, CAR, and CLMP are similar in structure to JAM-A, -B, and -C (contain two Ig-like domains, a single transmembrane domain, and C-terminal PDZ binding domain), they do contain distinguishing elements: the cytoplasmic tails are longer (105–118 residues in JAM-4, CAR, and CLMP vs. 40–50 residues in JAM-A, -B, and -C) and contain different subclasses of PDZ domain (PDZ1 in JAM-4 and CAR vs. PDZ 2 in JAM-A, -B, and –C). JAM-4 mRNA is weakly expressed in rat lung (Hirabayashi *et al*., 2003). CAR is identified by immunocytochemistry on the basolateral sides of non-permeabilized human airway epithelial cells (Walters *et al*., 1999). CLMP mRNA is expressed in human lung (Raschperger *et al*., 2004).

Though JAM-A has been the most thoroughly evaluated of the TJ-associated Ig proteins, its function at TJs remains unclear. As opposed to claudins (Furuse *et al*., 1998b), JAM-A does not induce TJ strands when expressed in fibroblasts (Itoh *et al*., 2001). JAM-A appears, nevertheless, to be linked to claudin-1 by ZO-1 (Ebnet *et al*. 2000) and MUPP-1 (Hamazaki *et al*., 2002) through PDZ domain interactions. Partitioning-defective protein (PAR)-3, a regulator of cell polarity, binds to JAM-A through its PDZ domain (Ebnet *et al*., 2003). Thus, it appears that JAM-A is tethered to claudins by protein adaptors and recruits PAR-3 to the TJ (Mizuno *et al*., 2003). PAR-3 is associated with atypical PKC and PAR-6 to form a complex (PAR-3/aPKC/PAR-6) that has been shown to facilitate TJ formation and establish cell polarity in mammalian epithelial cells (Ohno, 2001). Other cytoplasmic proteins bound to JAM-A include calcium/calmodulin-dependent serine protein kinase (CASK) (Martinez-Estrada *et al*., 2001) and cingulin (Bazzoni *et al*., 2000), which respectively, are suggested to serve signalling and tethering functions.

2.2.2 Adherens junctions

Farquhar and Palade described the AJ in epithelial cells as an electron-dense narrowing of the intercellular space to 25–35 nm located just below the TJ (Farquhar and Palade, 1963) (Figure 2.1). AJs hold epithelial cells together by tight calcium-dependent links. Three

principal proteins have been identified in AJs: cadherins, β-catenin, and α-catenin. Cadherins constitute a major class of adhesion molecules that supports homophilic cell–cell adhesion that is critical to the development and maintenance of all solid tissues (Takeichi, 1991). The first cadherin to be identified was epithelial (E)-cadherin (cadherin-1), a prototypical classical type I cadherin (Takeichi, 1995)[2,3] (Figure 2.2). Classical cadherin extracellular domains contain five tandem repeats of approximately 110 amino acids (cadherin domains) separated by four calcium-binding pockets. The N-terminus contains a conserved HAV motif required for homotypic binding between cadherins on neighbouring cells. The role of the remaining four tandem cadherin repeats in adhesive binding is not known. The cytoplasmic domains are 150–160 amino acids in length and mediate interactions with the actin cytoskeleton through linker proteins known as catenins (Takeichi, 1995). E-cadherin associates with the armadillo protein family member β-catenin, which then binds to α-catenin to form a roughly stoichiometric complex (Ozawa and Kemler, 1992).

AJs have long been believed to link actin cytoskeleton networks across cell–cell junctions through direct interactions between E-cadherin, β-catenin, α-catenin, and actin. However, existence of a quaternary complex containing these four species has never been demonstrated. Recent data suggest that α-catenin does not simultaneously bind to both actin and the E-cadherin–β-catenin complex. Furthermore, some evidence suggest that α-catenin may act as a molecular switch to actively regulate actin assembly at sites of E-cadherin-mediated cell–cell adhesion (Drees *et al*., 2005), thereby introducing more complexity to its known AJ protein linker function.

Pulmonary epithelial cells express E- and P-cadherin. In mouse embryonic lungs, E- and P-cadherin are expressed in all pulmonary epithelial cells during early development. P-cadherin gradually disappears, first from epithelium lining larger airway, then eventually from the remainder of lung (Hirai *et al*., 1989). Normal rat lung shows staining of E-cadherin predominantly in alveolar type II cells (Kasper *et al*., 1995). The human bronchial epithelial cell line, 16HBE14o(-), expresses E- and P-cadherin, but not N-cadherin. Increasing confluence of these cells in culture is associated with increased E-cadherin and decreased P-cadherin expression (West *et al*., 2002). In normal human bronchial epithelium, columnar cells express moderate levels of E-cadherin, while basal cells express high levels of P-cadherin (Smythe *et al*., 1999).

E-cadherin-β-catenin interactions have been suggested to be important in a variety of functions that may be relevant to pulmonary disease, including regulatory roles in protease-activated receptor (PAR)-2-mediated increase in airway epithelial permeability (Winter *et al*., 2006), epithelial proliferation and lung extracellular matrix (ECM) remodelling and repair in response to lung injury (Douglas *et al*., 2006), and as markers of and possibly mediators of lung cancer progression, state of differentiation, and metastatic potential (Awaya *et al*., 2005). β-catenin is also a component of the Wnt signalling pathway and serves as a transcriptional co-activator with T-cell factor/lymphocyte enhancer factor (Tcf/Lef) (Nelson and Nusse, 2004). Tcf/Lef has been implicated in the development of cancers, including lung cancer (Ohira *et al*., 2003).

---

[2] E-cadhein is also known as uvomorulin, Arc-1, liver cell adhesion molecule (L-CAM), and cell-CAM 120/80.

[3] Other classical type I cadherins include the desmosomal cadherins (desmoglein and desmocollin), neural (N)-cadherin (cadherin-2), placental (P)-cadherin (cadherin-3), and retinal (R)-cadherin (cadherin-4). Each assigned letter indicates the tissues in which the cadherin was originally identified.

2.2.3 Desmosomes

The desmosome, sometimes referred to as a 'spot-weld' between cells, is the third member of the tripartite junctional complex described by Farquhar and Palade (Farquhar and Palade, 1963) (Figure 2.1). Ultrastructurally, desmosomes measure between 0.1 and 1.5 μm in diameter and are delineated by an electron-dense plaque and electron-dense filaments that span the intercellular space (Cowin, 1985). In contrast to AJs, which are linked to actin filaments, desmosomes are linked to intermediate filaments (IFs). IFs are composed of tissue-specific complements of desmin, vimentin, and/or cytokeratins (Yamada *et al*., 1996). Cytokeratin expression is often used as a marker of epithelial cell morphology. In early states of human lung development, 'simple' cytokeratins (cytokeratin-7, -8, -18, -19) are detected in bronchial epithelial cells. At later stages, other cytokeratin isoforms (-13 and -14) can be detected, with differential expression in columnar vs. basal cells (Broers *et al*., 1989). Cytokeratin-19 expression (suggested to influence type II alveolar phenotype) in primary rat alveolar type II cells varies with factors influencing cell shape and intercellular contacts, e.g. lower cell seeding density and low calcium levels (decreases desmosome formation) (Paine *et al*., 1995). Vimentin is expressed in fetal bronchial epithelium, but decreases to a few scattered bronchial cells at birth and into adulthood. Desmin filaments are present in smooth muscle cells of the lung (Broers *et al*., 1989). IFs emanate from the desmosome plaques into the adjacent cytoplasm, looping repeatedly from the cytoplasm to the plaque and then back into the cytoplasm (Franke *et al*., 1983). In pseudostratified airway epithelium, desmosomes are present along lateral aspects of columnar cells, particularly towards cell apices, and at columnar-basal cell junctions.

The major constituents of desmosomes are membrane glycoproteins known as desmosomal cadherins. Desmosomal cadherins are single-pass, transmembrane-spanning proteins containing conserved regions of homology on the extracellular domain required for calcium binding and adhesion, and on the cytoplasmic domain required for binding to cytoplasmic adapter proteins. Two subclasses of desmosomal cadherins are known: the desmogleins (Dsg) and desmocollins (Dsc) (Figure 2.2). These proteins are encoded by individual genes that are clustered on human chromosome 18q12.1 (Hunt *et al*., 1999). Three Dsgs (Dsg-1, -2, and -3) and three Dscs (Dsc-1, -2, and -3) have been identified. These heterogeneous isoforms are expressed in tissue-, cell stratification-, and differentiation-specific patterns (Koch *et al*., 1992). The extracellular N-terminus of Dsg-1 has a short 29 amino acid propeptide and only four tandem repeat domains, compared to the longer propeptide and five cadherin repeats seen in other classical cadherins. Also, the adhesion motif, R/YAL, required for homophilic adhesion, differs from the classical cadherin HAV sequence (Kowalczyk *et al*., 1994).

Desmosomal cadherins are the pathophysiologic targets of autoimmune or toxin-mediated disruption in the human blistering skin diseases, pemphigus and bullous impetigo (including its generalized form, staphylococcal scalded skin syndrome) (Payne *et al*., 2004). Mutations in the human Dsg-1 gene have been linked to the rare autosomal dominant disorder striate palmoplantar keratoderma (SPPK), a disease characterized by marked hyperkeratotic bands on the palms and soles (Hunt *et al*., 2001). In the lung, immunohistochemical analysis shows variable expression of Dsg-3 in normal pulmonary epithelium, and in lung cancers (Boelens *et al*., 2007). Dsg-3 stains weakly at apical borders of basal bronchial epithelial cells and robustly at squamous cell carcinoma cell–cell junctions. Dsg-3 is not detected in lung adenocarcinomas. Negative Dsg-3 staining in lung cancer has been shown to be associated with decreased 5-year survival in non-small cell lung cancer, and to indicate poor prognosis in atypical pulmonary carcinoid tumours (Fukuoka *et al*., 2007).

The cytoplasmic plaque of desmosomes is complex and exhibits tissue-specific differences in both structure and composition. Members of two protein families populate the desmosomal plaque: armadillo proteins plakoglobin (PG) and the plakophilins (PPs), and plakin proteins desmoplakin (DP), envoplakin, periplakin, and plectin. All desmosomal plaque proteins are defined by structural motifs that participate in the coupling of desmosomal plaques to IFs (Troyanovsky and Leube, 1998). PG (also called γ-catenin) binds tightly to the cytoplasmic domains of Dsg and Dsc through highly-conserved sequence repeats known as Arm repeats (Troyanovsky *et al*., 1994a; Roh and Stanley 1995). Desmosomal cadherins lacking the PG binding site are unable to anchor IFs (Troyanovsky *et al*., 1994b). PG is not restricted to desmosomes – it also associates with AJ cadherins (Peifer *et al*., 1992), reflecting its close homology to β-catenin. In the lung, PG is highly expressed in normal bronchioles at apical and lateral borders of basal epithelial cells and in glandular epithelial cells (Boelens *et al*., 2007). Interestingly, PG has been shown, as has β-catenin, to be a component in Wnt signalling, and thus, has been suggested to have a role in cancer development. In non-small cell lung cancer cell lines, PG is weakly expressed, or absent. However, when PG expression is increased by treatment with a histone deacetylase inhibitor, Tcf/Lef transcription factor activity is reduced, which correlates with inhibition of cell growth and decreased malignant potential (Winn *et al*., 2002).

The PPs are desmosomal plaque proteins that localize to both the desmosome and to the nucleus. PP-1, originally named ‘band 6 protein’, was isolated as an accessory protein bound to keratin in stratified and complex epithelia (Heid *et al*., 1994). Two additional splice variants have been cloned, PP-2, and PP-3 (Bonne *et al*., 1999; Mertens *et al*., 1999). PPs are composed of an N-terminal head domain and a C-terminal domain containing 9 Arm repeats (Choi and Weis, 2005). The head domains mediate interactions with desmosomal proteins, including DP, PG, Dsg, and Dsc, and are sufficient to direct PPs to cell junctions (Kowalczyk *et al*., 1999).

The plakins are a family of large cytolinker proteins (200–700 kDa) that are important for coupling different adhesive junctions (desmosomes, hemidesmosomes, and focal adhesion contacts) to the cytoskeleton. Seven plakin family members have been identified based on domain structure. Four of these, DP, plectin, envoplakin, and periplakin, have been localized to desmosomes (Jefferson *et al*., 2004).

DP is the most abundant of desmosomal plaque proteins. It is expressed in two splice variant isoforms (DP-1 and -2) and is required both for assembly of desmosomes and for their association with IFs. The N-terminal plakin domain peptide (DP-NTP) targets DP to desmosomal plaques (Bornslaeger *et al*., 1996). The C-terminal domain of DP contains three plakin repeat domain (PRDs). DP PRD crystal structure shows that each repeat contains 4.5 copies of a 38 amino acid motif that forms a globular structure containing a conserved basic groove that may represent an IF binding site (Choi *et al*., 2002). Combined PRDs support a strong bond with vimentin (individual PRDs weakly bind to vimentin). Plectin, envoplakin, and periplakin do not appear to play major roles in desmosome function.

In normal lung, DP stains weakly at the basolateral borders of suprabasal cells and at the apical ends of ciliated cells. DP is highly expressed in bronchial glands (Young *et al*., 2002). DP expression has been associated with pulmonary epithelial transdifferentiation and has been shown to vary with lung cancer cell type (Boelens *et al*., 2007). DP expression at cell–cell junctions is high in adenocarcinomas, and particularly high in squamous cell carcinomas. Furthermore, microarray analyses also reveal significant differential gene expression of DPs (as well as cytokeratin-18) between different lung cancer types (Young *et al*., 2002).

2.3 Cell–substratum adhesion

2.3.1 Integrins

The principal adhesion molecules involved in epithelial cell binding to the basement membrane are members of the integrin family. In humans, there are 18α and 8β integrin subunits that noncovalently associate to form 24 heterodimeric pairs. The crystal structure for the extracellular domains of an intact integrin (Xiong *et al*., 2002) demonstrates the presence of a cation-binding site in the exposed β subunit face that coordinates all but one of the free sites on bound cation, leaving a free coordination site to interact with negatively charged residues. Recognition sequences on integrin ligands contain a corresponding negatively charged amino acid, e.g. aspartic acid in the arginine-glycine-aspartic acid (RGD) sequence. RGD is recognized by a substantial subset of integrins. The closely-apposed α subunit helps determine ligand binding specificity; for example, a subset of α subunits contains an inserted (I) domain that extends from the α subunit face to form cooperative α/β ligand binding sites (Lee *et al*., 1995).

At least seven different integrins (α2β1, α3β1, α6β4, α9β1, αvβ5, αvβ6, and αvβ8) are expressed in airway epithelial cells of healthy adults (Damjanovich *et al*., 1992). These integrins, and some of their known ligands, are listed in Table 2.1. Of these, α3β1 and α6β4

Table 2.1 Integrins expressed on airway epithelial cells. VCAM, vascular cell adhesion molecule; L1-CAM, L1 cell adhesion molecule; vWF, von Willebrand factor; ADAMs, a disintegrin and metalloprotease protein; LAP, latency-associated peptide; TGF, transforming growth factor

Integrin	Known ligand(s)	Distribution
α2β1	Collagen I (IV), Tenascin C, Echovirus	Diffusely expressed, principally on basal cells
α3β1	Laminin-5, -10, -11	Diffusely expressed with highest level expression on basal surface of basal cells
α6β4	Laminin-5, -10, -11	Restricted to basal surface of basal cells
α9β1	Tenascin C, Osteopontin, VCAM-1, L1-CAM, vWF, Factor XIII, Tissue Transglutaminase, Fibronectin EIIIA Domain, Angiostatin, ADAMs 1,2,3,9,15 (at least)	Diffusely expressed, principally on basal cells
α5β1	Fibronectin	Diffusely expressed, but only after injury
αvβ5	Vitronectin, Adenovirus, Ostepontin	Diffusely expressed, principally on basal cells
αvβ6	LAP of TGF-β1 and TGF-β3, Fibronectin, Tenascin C, Osteopontin, Vitronectin, Foot and Mouth Disease Virus	Diffusely expressed, principally on basal cells
αvβ8	LAP of TGF-β1 and TGF-β3, Vitronectin	Diffusely expressed on basal cells

are the only receptors for matrix proteins known to be present in normal epithelial basement membranes (laminin-5, -10, and -11) (Carter *et al*., 1991; Eble *et al*., 1998) (Figure 2.2). α3β1 is concentrated at epithelial cell bases and also (with lower expression levels) at lateral and apical surfaces (unpublished observations). Mice lacking the α3 subunit have defects in branching morphogenesis in the lung and kidney (Kreidberg *et al*., 1996). Furthermore, these animals have dramatic defects in the structural organization of epithelial basement membranes (DiPersio *et al*., 1997). These observations led to identification of a role for α3β1 in organizing basement membranes into ordered structures. Studies with isolated cells from α3-deficient mice demonstrated an important role for α3β1 in epithelial cell migration (Hodivala-Dilke *et al*., 1998). α6β4 is restricted to the cell–substratum surface of basal cells, where it serves as a major component of hemidesmosomes (Stepp *et al*., 1990) (discussed below). α2β1 is thought to interact with collagen IV (a common basement membrane constituent), but its preferred ligands are other collagen isoforms, e.g. collagen I (Kern and Marcantonio, 1998). Furthermore, diffuse surface expression of α2β1 suggests other functions, and possibly other biologically important ligands. While α2β1 and α3β1 have been suggested to play roles in homotypic cell–cell interactions in epithelia (Carter *et al*., 1990), this has not been demonstrated in experimental models (Weitzman *et al*., 1995). Furthermore, mice lacking either α3 or α2 have not been described to have defects in epithelial cell–cell interactions (Kreidberg *et al*., 1996; Holtkotter *et al*., 2002).

The other integrins that are expressed on basal airway epithelial cells, α5β1 (the original 'fibronectin receptor'), α9β1, αvβ5, αvβ6, and αvβ8, recognize a wide array of ligands that are not components of healthy epithelial basement membranes. Many of the ligands recognized by these integrins (e.g., fibronectin, tenascin C, and osteopontin) are among the most highly-induced proteins at sites of epithelial injury (Young *et al*., 1994; Weinacker *et al*., 1995). Vitronectin, the best characterized ligand for αvβ5, is principally a plasma protein and therefore, is also likely to be enriched in the airway after injury or other increases in vascular permeability. In addition, α5β1, which is generally not present in healthy adult airway epithelium in vivo, is rapidly induced by epithelial injury (Pilewski *et al*., 1997) (Figure 2.3). Thus, these integrins appear to be good candidates to serve as sensors that allow epithelial cells to rapidly detect and respond to ECM changes that accompany lung and airway inflammation and injury.

Integrins as regulators of cell proliferation

Integrins play critical roles in regulating cell proliferation (Guadagno *et al*., 1993). Most adherent cells are incapable of proliferating without signals from the ECM that are transmitted through integrins. Integrins are frequently enriched within membrane microdomains containing other cell surface receptors (e.g., growth factor receptors) that contribute to cell proliferation. These microdomains include structures called focal adhesions (FAs) (Figure 2.1), regions of close apposition to the underlying matrix organized around links between integrins and the ends of actin filaments. FAs contain large numbers of adaptor proteins, signalling kinases, and other signalling pathway components. Although signals initiated by integrins have been shown to enhance cell proliferation in vitro without addition of exogenous, soluble growth factors, it is likely that these results are explained, in part, by autologous production of growth factors by the cultured cells. Ligation of integrins can activate several kinases known to be activated by growth factor receptors, including Src, Ras (Schlaepfer *et al*., 1994), and mitogen-activated protein (MAP) kinases (Chen *et al*., 1994).

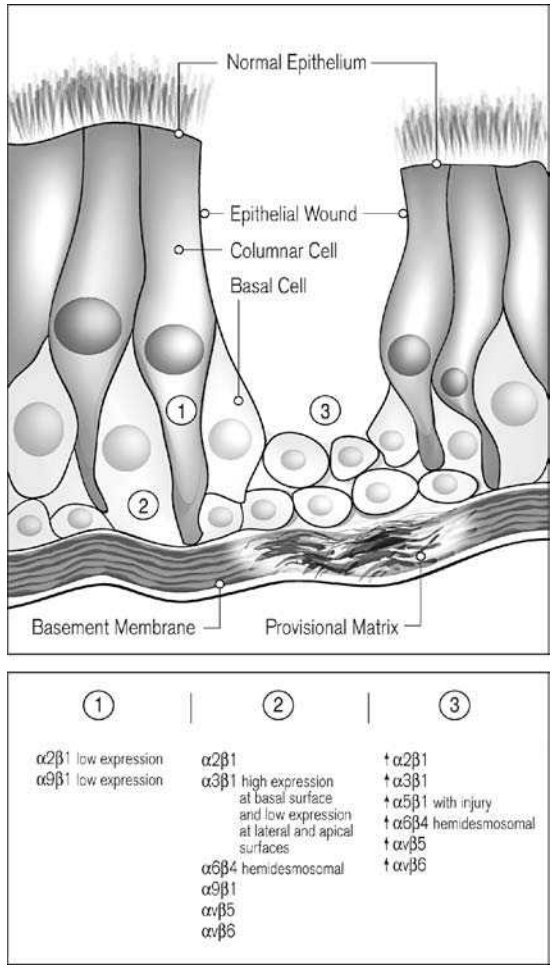


Figure 2.3 Changes in the level and distribution of airway epithelial integrin expression in response to injury. Depicted are normal, uninjured epithelium (Normal Epithelium), and a theoretical site of denudation (Epithelial Wound). Differential integrin expression is seen between (1) normal columnar and (2) basal cells, and (3) epithelial cells at the wound site associated with a provisional extracellular matrix. $\alpha 6\beta 4$ is restricted to the cell–substratum surface of basal cells, where it serves as a major component of hemidesmosomes. $\alpha 3\beta 1$ is concentrated at the basal surface, but is also expressed at lower levels around the lateral and apical surfaces of cells throughout the epithelium. $\alpha 5\beta 1$ is expressed only at the injury site. Expression of $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 4$, $\alpha v\beta 5$, and $\alpha v\beta 6$ are dramatically upregulated along the injured surface

A specific subset of integrins (including $\alpha 1\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$) can induce, or enhance, cell proliferation through interaction of the α subunit cytoplasmic domain with caveolin-1, a membrane protein that plays a role in organizing membrane microdomains. In this pathway, caveolin-1 recruits the Src family kinases, Yes or Fyn, which recruit the adaptor protein Shc, that in turn leads to recruitment and activation of the well-characterized Ras pathway (Wary *et al*., 1998).

Transgenic mice expressing β1, alone or in combination with subunits α2 or α5, in suprabasal keratinocytes exhibit epidermal hyper-proliferation, a prominent feature of the human skin disease psoriasis (Carroll *et al*., 1995). However, several studies have also suggested that overexpression of α5β1 can inhibit cell proliferation (Giancotti and Ruoslahti, 1990; Varner *et al*., 1995). One mechanism by which such inhibition occurs has been demonstrated in studies utilizing the colon carcinoma cell line HT-29, a cell line that normally does not express α5β1. Heterologous expression of α5β1 in these cells diminished their proliferative capacity, an effect that appeared to involve cellular quiescence induced by the growth arrest specific gene *gas-1* (Varner *et al*., 1995). Interestingly, this effect was a consequence of expression of unligated integrin, since plating of transfected cells on dishes coated with the α5β1 ligand fibronectin reversed *gas-1* induction and growth inhibition. If a similar pathway is operative in normal epithelial cells, the combined effects of the growth-promoting role of ligated integrin and the growth inhibitory role of unligated integrin would provide an elegant mechanism by which cells in normal adult epithelia (which would not be in contact with fibronectin) are kept out of the cell cycle, while cells at sites of injury (where fibronectin in greatly enriched) can be stimulated to proliferate.

Integrins as regulators of epithelial cell survival

Nontransformed epithelial cells cannot survive in the absence of anchorage to the ECM and die by apoptosis soon after detachment, a process that has been termed anoikis (Frisch and Francis, 1994). This process, like the withdrawal of growth and survival factors from other primary cells, is mediated, at least in part, by activation of a cascade of caspase proteases that lead to rapid and efficient cell death. Epithelial cells are thus primed to activate a classical caspase-mediated execution program; ligated integrins appear to deactivate this program. Anoikis likely plays the important role of preventing detached epithelial cells in hollow organs such as the lung or gastrointestinal tract from reattaching at inappropriate sites. However, anoikis does not appear to be a universal feature of all epithelia. For example, rather than die in this absence of input from integrins, keratinocytes terminally differentiate and begin the process of keratinization (Watt, 2002). In the mammary gland, where involution is a normal phenomenon that follows termination of breast-feeding, apoptosis in the involuting gland is associated with degradation of the stromal matrix by metalloproteinases, a process that presumably results in unligated integrins. Indeed, in this system, apoptosis can be induced either by antibodies to β1 integrins or by overexpression of the matrix-degrading protease stromelysin-1 in the absence of obvious cell detachment (Boudreau *et al*., 1995).

Role of integrins in epithelial cell polarity

In vivo, surface epithelial cells, including those lining the conducting airway and alveoli of the lung, are polarized and establish specialized structures along their basal, lateral, and apical surfaces. Establishment of appropriate epithelial polarity requires input from integrins (Ojakian and Schwimmer, 1994). In most epithelia, normal polarity is principally dependent on interactions between integrins and the basement membrane constituent laminin (Sorokin *et al*., 1990). Furthermore, many of the normal differentiated functions of epithelial cells cannot be induced in nonpolarized cultures (Streuli *et al*., 1991). Considerable insight into

the mechanisms underlying establishment of polarity has come from studies of mammary gland epithelial cells. In tissue culture, these cells can be induced to form polarized glandlike structures with a central lumen by overlaying cultures with epithelial-derived basement membrane proteins, or with purified laminin (Muschler *et al*., 1999). Only under these circumstances will mammary epithelial cells fully differentiate in response to lactogenic hormones. These responses to laminin are mediated by input from both α3β1 and α6β4, as well as other nonintegrin receptors (Muschler *et al*., 1999).

Renal epithelial cells (MDCKs) can also be induced to form polarized structures containing an apical epithelium facing a lumen if they are plated in a three-dimensional culture environment. In this case, MDCK cells produce their own laminin and organize it into a basement membrane along the basal surface utilizing α3β1. Studies utilizing inducible forms of the small GTPase Rac1 have demonstrated a critical role for Rac1-induced reorganization of the actin cytoskeleton in establishing epithelial polarity (O'Brien *et al*., 2001). A similar role for polarity in secretory cell differentiation has been demonstrated in serous cells derived from airway submucosal glands, which require input from a β1-integrin and laminin to express their differentiated secretory cell phenotype (Tournier *et al*., 1992).

Integrin-mediated activation of transforming growth factor (TGF)-β

As noted above, integrins can interact with a wide variety of extracellular ligands. Although integrin ligation is usually thought of as a mechanism that induces signals in the integrin-expressing cell, it has recently been recognized that integrins can also modify the conformation of extracellular ligands. One of the most dramatic examples of this is integrin-mediated activation of TGF-β. TGF-β is secreted as a latent complex composed of the mature cytokine and an N-terminal fragment of the same gene product assembled as a noncovalently-associated double homodimer. In this form, TGF-β is unable to bind to its receptors and is therefore considered latent. Most tissues, including the lung, contain large amounts of this latent complex that is stored and chemically cross-linked to components of the ECM. Thus, much of the regulation of the biological effects of TGF-β involves extracellular activation of these latent complexes. At least two integrins that are expressed on lung epithelial cells, αvβ6 (Munger *et al*., 1999) and αvβ8 (Mu *et al*., 2002), bind to the latency associated peptide of TGF-β1 and TGF-β3 and can induce activation of latent complexes. In the case of αvβ6, this pathway has been shown to be critically important in in vivo models of pulmonary fibrosis (Munger *et al*., 1999) and acute lung injury (Pittet *et al*., 2001). Mice deficient in the αvβ6 integrin develop low-grade pulmonary inflammation and macrophage activation, suggesting that this integrin plays a critical role in maintaining normal lung homeostasis. Rescue experiments showed that limited transgenic expression of αvβ6 in a subset of alveolar epithelial cells was sufficient to prevent pulmonary inflammation and macrophage activation (Huang *et al*., 1998). Lifelong absence of αvβ6 results in persistent over-expression of matrix metalloproteinase (MMP)-12 in alveolar macrophages, and eventual development of age-related emphysema (Morris *et al*., 2003). This suggests that acquired or inherited abnormalities in αvβ6, TGF-β, and other components of this signalling pathway could contribute to the development of emphysema. αvβ6 also appears to play a critical role in maintaining the normally blunted inflammatory response in alveolar epithelial cells; this process can be transiently overcome by ligation of toll-like receptors on macrophages (e.g. in response to alveolar infection), a process that involves rapid down-regulation of αvβ6 expression (Takabayshi *et al*., 2006). All of the effects described above appear to be

regulated by αvβ6 expressed on alveolar epithelial cells. The in vivo significance of αvβ6 and αvβ8 that is expressed on epithelial cells in the conducting airway is less clear. αvβ8 has been shown to play an important role in inhibiting the proliferation of cultured airway epithelial cells, an effect that appears to be due to its ability to activate TGF-β (Fjellbirkeland *et al*., 2003).

Integrins in repair of wounded epithelia

Surface epithelia all have the capacity to repair areas of denudation (Chapter 4). This process involves at least three functional changes in epithelial cells involved in repair: spreading, migration, and proliferation. Each of these processes requires integrins. The effects of wounding on local expression of integrins and their ligands have been most extensively studied in squamous epithelia, such as the skin. Cutaneous wounds contain a provisional matrix that is rich in the integrin ligands fibronectin, osteopontin, and tenascin. In response to epithelial injury, there are dramatic changes in both the spatial distribution and level of expression of epithelial integrins. Expression of α2β1, α3β1, α5β1, α6β4, αvβ5, and αvβ6 are dramatically upregulated along the injured surface (Larjava *et al*., 1993) (Figure 2.3). Tightly-regulated spatial and temporal patterns of expression for each of these integrins suggest that each might play a unique role in orchestrating normal healing. However, there must also be substantial redundancy in this process, since inactivation of single integrins (e.g., αvβ5 (Huang *et al*., 2000) or αvβ6) or even two integrins simultaneously (e.g., αvβ5 and αvβ6; unpublished observations) does not lead to significant impairment in the rate or quality of cutaneous wound healing.

The most careful study of the effects of airway epithelial wounding on integrin expression was performed utilizing human bronchial grafts placed under the skin of severe combined immune deficiency (SCID) mice (Pilewski *et al*., 1997). In this system, the pattern of integrin expression seen in the absence of injury was quite similar to the pattern seen in normal human airway. After injury, the most prominent changes were upregulation of α5β1, αvβ5, and αvβ6 expression along the wound edge. As in cutaneous wounds, α2β1 and α6-containing integrins were diffusely expressed on cells above the basal layer. The relevance of these findings to in vivo injury in humans was confirmed by the observation that integrin expression in airway from patients with cystic fibrosis was similar to that seen in the injured xenografts (Pilewski *et al*., 1997).

In vitro studies have also identified a role of integrin-mediated TGF-β activation in sheet migration and closure of wounded airway epithelium. Both αvβ6 and αvβ8 can be 'activated' by mechanical scratch wounds of cultured airway epithelial cells and contribute to activation of locally produced TGF-β1 (Neurohr *et al*., 2006). Under these conditions, TGF-β inhibits the rate of sheet migration and wound closure independent of any effects on cell proliferation. Interestingly, blockade of αvβ8 enhances the rate of wound closure under these conditions, whereas blockade of αvβ6 has no effect. This appears to be due to a TGF-β-independent role of αvβ6 in accelerating the rate of epithelial sheet migration.

Roles of integrins in epithelial neoplasia

Initial efforts to understand the roles that integrins might play in the development of epithelial tumours involved descriptive immunohistochemistry. Descriptions of integrin staining

in lung cancers, for example, have shown qualitatively similar integrin repertoires between squamous, adeno-, and large cell carcinomas and normal bronchial epithelium, while bronchioloalveolar carcinoma (BAC) integrin expression paralleled that of the alveolar epithelium (e.g. by its strong expression of α1β1 and α3β1) (Koukoulis *et al*., 1997). However, it soon became apparent that the changes in integrin protein expression in epithelial tumours are complex and heterogeneous. As noted above, many primary epithelial cells undergo apoptosis in response to loss of integrin ligation. However, in most epithelial cancers, loss of integrin ligation does not induce apoptosis, suggesting that carcinomas do not require input from integrins to survive. Similarly, carcinomas do not appear to require anchorage (i.e., integrin dependence) for growth. By anchoring epithelial cells to the normal basement membrane, integrins could impede tumour cell migration and subsequent invasion and metastasis. It is not surprising, therefore, that a general decrease in integrin expression is seen in many invasive carcinomas. However, since integrins can also enhance growth factor-mediated proliferation, provide traction for cell migration through the ECM, and localize matrix-degrading proteases to the leading edge of migrating cells (Brooks *et al*., 1996), it is also not surprising that many epithelial tumours utilize integrins to enhance their growth and/or invasion. In at least one case, the same integrin (α6β4) that restricts movement of normal epithelial cells through hemidesmosome anchorage is utilized by malignant epithelial cells to support migration and invasion (Chao *et al*., 1996; Gambaletta *et al*., 2000). In this case, a key step is the redistribution of α6β4 from hemidesmosomes to the leading edge, an otherwise normal homeostatic mechanism that supports migration of epithelial cells at the wound edges.

The integrin αvβ6 also appears to modify malignant transformation and enhance the growth and invasion of epithelial tumors (Xue *et al*., 2001). In this case, αvβ6 affects both functions through different mechanisms. In the early stages of malignant transformation the cytoplasmic domain of the β6 subunit specifically supports tumour cell proliferation, both in vivo and in vitro, and also induces expression of the metalloprotease MMP-9 (Thomas *et al*., 2001), which enhances tumor cell invasion.

2.3.2 Hemidesmosomes

Hemidesmosomes are specialized junction structures that mediate epithelial cell–substratum adhesion in stratified squamous, transitional, and pseudostratified epithelia. Hemidesmosomal ultrastructure reveals small electron-dense domains in the plasma membrane composed of an inner and an outer plaque, and a sub-basal dense plate (Figure 2.1). The inner plaque serves as an anchorage site for intracellular IFs. Hemidesmosomes are best described in the skin, where they provide stable adhesion of the epidermis to the underlying dermis, conferring resistance to mechanical stress (Borradori and Sonnenberg, 1999).

Although hemidesmosomes, like desmosomes, are linked to the IF system, they do not contain desmosomal proteins such as PG, desmosomal cadherins Dsg and Dsc, plakophilins (PPs), or the plakins DP, envoplakin, or periplakin. They do, however, contain integrin α6β4, the type XVII collagen bullous pemphigoid antigen (BP)180, the tetraspanin, CD151, and two plakin family members plectin and BP230.

Integrin α6β4 links hemidesmosomes to a major component of the basement membrane, laminin-5 (also referred to as laminin-322), whereas plectin and BP230 link hemidesmosomes to IFs. Mice homozygous for null mutations of either the α6 or the β4 subunit die soon after birth with severe blistering of the skin (Georges-Labouesse *et al*., 1996; van der Neut *et al*.,

1996). A severe blistering disease has also been identified in a human infant homozygous for a mutation of the α6 subunit (Ruzzi *et al*., 1997). Two mutations identified in the β4 gene of patients with a nonlethal form of junctional epidermolysis bullosa (JEB) disrupt its binding to plectin (Rezniczek *et al*., 1998). When β4 cannot bind to plectin, neither BP180 nor BP230 are efficiently recruited into hemidesmosomes (Schaapveld *et al*., 1998). Plectin gene mutations have also been associated with human skin fragility, though not to the degree observed in plectin-deficient mice (Andra *et al*., 1997; Koss-Harnes *et al*., 2002).

Another laminin-binding integrin on human keratinocytes, α3β1, is strongly associated with CD151, with which it forms 'pre-hemidesmosomal' clusters at the basal cell surface (Sterk *et al*., 2000). CD151 then binds to α6 to become a component of mature hemidesmosomes, while α3β1 is recruited into FAs or redistributed to cell–cell contacts. Although α3β1 does not appear to directly participate in hemidesmosome assembly, it might, together with other β1-containing integrins, contribute to their formation by affecting localization of α6β4. In β1-deficient mice, hemidesmosome numbers are reduced, which seems to correlate with their observed phenotype of skin thickening and blistering (Brakebusch *et al*., 2000).

Regulated hemidesmosome disassembly is thought to be important in cellular processes including cell migration and differentiation. Proposed regulatory mechanisms for disassembly of kertinocyte hemidesmosomes include epidermal growth factor-(EGF)-induced phosphorylation of β4 by Fyn (a pathway that was shown to regulate experimental metastases formation) (Mariotti *et al*., 2001) and β4 phosphorylation by PKCα (and possibly other kinases), resulting in loss of interaction with plectin (Rabinovitz *et al*., 2004).

Previously, it was thought that pulmonary epithelial hemidesmosomes occurred exclusively between basal cells and the underlying basement membrane; the few columnar cells reaching down to the basement membrane were thought to be anchored in place, rather, through desmosomal attachments to the basal cells (Michelson *et al*., 2000). More recently, normal human bronchial epithelial (NHBE) cells have been shown to express α6β4, hemidesmosome-associated structural proteins bullous pemphigoid antigen (BPAG)-1 and -2, and to produce laminin-5. Bronchial biopsy specimens have also been shown to contain laminin-5 in their basement membranes, and BP230, BP180, and α6β4 at epithelial cell-ECM junctions. Furthermore, ultrastructural imaging has revealed structures resembling intact hemidesmosomes (Michelson *et al*., 2000).

2.4 Conclusion

The pulmonary epithelium is no longer known simply as a passive protective barrier. It is now recognized as a highly organized, multifunctional tissue that plays critical roles in normal and pathologic function throughout the entire respiratory system. Epithelial structures first visualized over a century ago and dramatically revisited by EM in the 1960s spawned an important and expanding area of research – defining epithelial cell–cell and cell–substratum interactions. Significant advances in knowledge have been made in the identification and characterization of critical adhesion structures including TJs, AJs, desmosomes, and hemidesmosomes and specific adhesion molecules like integrins. These structures and their functional components have since been shown to have critical roles in myriad functions including maintenance of epithelial cell differentiation, proliferation, repair, polarity, paracellular barrier function and ion selectivity, regulating organ morphogenesis and repair, and

determining tumour malignant and metastatic potential. Many of these structures have now been identified in the pulmonary epithelium. Elucidating details of these adhesion structures and molecules and their functions promises to provide wide-ranging insights into pulmonary health and disease.

Acknowledgement

We thank Lisa Hsiao for figure artwork.

Abbreviations

TJ, tight junction; AJ, adherens junction; EM, electron microscopy; P, protoplasmic; E, exoplasmic; JAM (junctional adhesion molecule); N, amino terminus; C, carboxyl terminus; MDCK, Madin–Darby canine kidney cells; TM4-, transmembrane domain 4-deficient isoform; CK, casein kinase; PKC, protein kinase C; ZO, zonula occludens, VAMP, vesicle-associated membrane protein; VAP33, VAMP-associated protein of 33 kDa; MAGUK, membrane-associated guanylate kinase; SH, src homology; PDZ, Post-synaptic density protein-Drosophila disc large tumour suppressor-ZO-1 protein; GK, guanylate kinase; MUPP, multi-PDZ domain protein-1; PALS, Protein Associated with Lin Seven; PATJ, PALS-1-associated protein; EGF, epidermal growth factor; S1P, sphingosine 1-phosphate; TNF, tumour necrosis factor; EMT, epidermal mesechymal transition; CAR, coxsackie and adenovirus receptor; CLMP, CAR-like membrane protein; ESAM, endothelial cell-selective adhesion molecule; PAR-3, partitioning-defective protein-3; CASK, calcium/calmodulin-dependent serine protein kinase; L-CAM, liver cell adhesion molecule; PAR-2, protease-activated receptor-2; ECM, extracellular matrix; IF, intermediate filament; Tcf/Lef, T-cell factor/lymphocyte enhancer factor; Dsg, desmogleins; Dsc, desmocollins; SPPK, striate palmoplantar keratoderma; PG, plakoglobin; PP, plakophilin; DP, desmoplakin; DP-NTP, desmoplakin N-terminal plakin domain peptide; PRD, plakin repeat domain; RGD, arginine-glycine-aspartic acid; I, inserted; SCID, severe combined immune deficiency; FA, focal adhesion; MAP, mitogen-activated protein; TGF, transforming growth factor-β; MMP, matrix metalloproteinase; BAC, bronchioloalveolar carcinoma; MMP, metalloprotease; BP, bullous pemphigoid; FNIII, fibronectin type III; JEB, junctional epidermolysis bullosa; EGF, epidermal growth factor; NHBE, normal human bronchial epithelial cell; BPAG, bullous pemphigoid antigen.

References

Ando-Akatsuka, Y., Saitou, M., Hirase, T., Kishi, M., Sakakibara, A., Itoh, M., Yonemura, S., Furuse, M. and Tsukita, S. (1996). Interspecies diversity of the occludin sequence: cDNA cloning of human, mouse, dog, and rat-kangaroo homologues. *J Cell Biol* **133**, 43–47.

Andra, K., Lassmann, H., Bittner, R., Shorny, S., Fässler, R., Propst, F. and Wiche, G. (1997). Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev* **11**, 3143–3156.

Andreeva, A. Y., Krause, E., Müller, E.C., Blasig, I.E. and Utepbergenov, D.I. (2001). Protein kinase C regulates the phosphorylation and cellular localization of occludin. *J Biol Chem* **276**, 38480–38486.

Aurrand-Lions, M., Duncan, L., Ballestrem, C. and Imhof, B.A. (2001). JAM-2, a novel immunoglobulin superfamily molecule, expressed by endothelial and lymphatic cells. *J Biol Chem* **276**, 2733–2741.

Awaya, H., Takeshima, Y., Amatya, V.J., Ishida, H., Yamasaki, M., Kohno, N. and Inai, K. (2005). Loss of expression of E-cadherin and beta-catenin is associated with progression of pulmonary adenocarcinoma. *Pathol Int* **55**, 14–18.

Bartels, H. (1979). The air–blood barrier in the human lung. A freeze-fracture study. *Cell Tissue Res* **198**, 269–285.

Bazzoni, G., Martinez-Estrada, O.M., Orsenigo, F., Cordenonsi, M., Citi, S. and Dejana, E. (2000). Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *J Biol Chem* **275**, 20520–20526.

Bizzozero, T. (1870). Osservazioni sulla struttura degli epitheli pavimentosi stratificati. *Rend 1st Lomb Accad Sci Lett* Ser. II, 675.

Boelens, M. C., van den Berg, A., Vogelzang, I., Wesseling, J., Postma, D.S., Timens, W. and Groen, H.J. (2007). Differential expression and distribution of epithelial adhesion molecules in non-small cell lung cancer and normal bronchus. *J Clin Pathol* **60**, 608–614.

Bonne, S., van Hengel, J., Nollet, F., Kools, P. and van Roy, F. (1999). Plakophilin-3, a novel armadillo-like protein present in nuclei and desmosomes of epithelial cells. *J Cell Sci* **112**, 2265–2276.

Bornslaeger, E. A., Corcoran, C.M., Stappenbeck, T.S. and Green, K.J. (1996). Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell–cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. *J Cell Biol* **134**, 985–1001.

Borradori, L. and Sonnenberg, A. (1999). Structure and function of hemidesmosomes: more than simple adhesion complexes. *J Invest Dermatol* **112**, 411–418.

Boudreau, N., Sympson, C.J., Werb, Z. and Bissell, M.J. (1995). Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* **267**, 891–893.

Brakebusch, C., Grose, R., Quondamatteo, F., Ramirez, A., Jorcano, J.L., Pirro, A., Svensson, M., Herken, R., Sasaki, T., Timpl, R., Werner, S. and Fassler, R. (2000). Skin and hair follicle integrity is crucially dependent on beta 1 integrin expression on keratinocytes. *Embo J* **19**, 3990–4003.

Broers, J.L., de Leij, L., Rot, M.K., ter Haar, A., Lane, E.B., Leigh, I.M., Wagenaar, S.S., Vooijs, G.P. and Ramaekers, F.C. (1989). Expression of intermediate filament proteins in fetal and adult human lung tissues. *Differentiation* **40**, 119–128.

Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T. L., Aimes, R.T., Stetler-Stevenson, W.G., Quigley, J.P. and Cheresh, D.A. (1996). Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* **85**, 683–693.

Carroll, J. M., Romero, M.R. and Watt, F.M. (1995). Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. *Cell* **83**, 957–968.

Carter W.G., Wayner, E.A., Bouchard, T.S. and Kaur, P. (1990). The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell–cell and cell–substrate adhesion of human epidermal cells. *J Cell Biol* **110**, 1387–1404.

Carter, W. G., Ryan, M.C. and Gahr, P.J. (1991). Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. *Cell* **65**, 599–610.

Chao, C., Lotz, M.M., Clarke, A.C. and Mercurio, A.M. (1996). A function for the integrin alpha6beta4 in the invasive properties of colorectal carcinoma cells. *Cancer Res* **56**, 4811–4819.

Chen Q., Kinch, M.S., Lin, T.H., Burridge, K. and Juliano, R.L.. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J Biol Chem* **269**, 26602–26605.

Chen S.P., Zhou, B., Willis, B.C., Sandoval, A.J., Liebler, J.M., Kim, K.J., Ann, D.K., Crandall, E.D. and Borok, Z. (2005). Effects of transdifferentiation and EGF on claudin isoform expression in alveolar epithelial cells. *J Appl Physiol* **98**, 322–328.

Chen Y.H., Lu, Q., Goodenough, D.A. and Jeansonne, B. (2002). Nonreceptor tyrosine kinase c-Yes interacts with occludin during tight junction formation in canine kidney epithelial cells. *Mol Biol Cell* **13**, 1227–1237.

Choi, H.J. and Weis, W.I. (2005). Structure of the armadillo repeat domain of plakophilin 1. *J Mol Biol* **346**, 367–376.

Choi, H.J., Park-Snyder, S., Pascoe, L.T., Green, K.J. and Weis, W.I. (2002). Structures of two intermediate filament-binding fragments of desmoplakin reveal a unique repeat motif structure. *Nat Struct Biol* **9**, 612–620.

Citi, S., Sabanay, H., Jakes, R., Geiger, B. and Kendrick-Jones, J. (1988). Cingulin, a new peripheral component of tight junctions. *Nature* **333**, 272–276.

Cohen, C.J., Shieh, J.T., Pickles, R.J., Okegawa, T., Hsieh, J.T. and Bergelson, J.M. (2001). The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A* **98**, 15191–15196.

Colegio, O.R., Van Itallie, C., Rahner, C. and Anderson, J.M. (2003). Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. *Am J Physiol Cell Physiol* **284**, C1346–C1354.

Cowin, P., Grund, W. W. F. C., Kapprell, H-P and Karenbeck, J. (1985). *The Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants*. New York, John Wiley.

Coyne, C.B., Gambling, T.M., Boucher, R.C., Carson, J.L. and Johnson, L.G. (2003). Role of claudin interactions in airway tight junctional permeability. *Am J Physiol Lung Cell Mol Physiol* **285**, L1166–L1178.

Damjanovich, L., Albelda, S.M., Mette, S.A. and Buck, C.A. (1992). Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. *Am J Respir Cell Mol Biol* **6**, 197–206.

Del Maschio, A., De Luigi, A., Martin-Padura, I., Brockhaus, M., Bartfai, T., Fruscella, P., Adorini, L., Martino, G., Furlan, R., De Simoni, M.G. and Dejana, E. (1999). Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). *J Exp Med* **190**, 1351–1356.

DiPersio, C. M., Hodivala-Dilke, K.M., Jaenisch, R., Kreidberg, J.A. and Hynes, R.O. (1997). alpha3beta1 Integrin is required for normal development of the epidermal basement membrane. *J Cell Biol* **137**, 729–742.

Douglas, I.S., Diaz del Valle, F., Winn, R.A. and Voelkel, N.F. (2006). Beta-catenin in the fibroproliferative response to acute lung injury. *Am J Respir Cell Mol Biol* **34**, 274–285.

Drees, F., Pokutta, S., Yamada, S., Nelson, W.J. and Weis, W.I. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* **123**, 903–915.

Eble, J.A., Wucherpfennig, K.W., Gauthier, L., Dersch, P., Krukonis, E., Isberg, R.R. and Hemler, M.E. (1998). Recombinant soluble human alpha 3 beta 1 integrin: purification, processing, regulation, and specific binding to laminin-5 and invasin in a mutually exclusive manner. *Biochemistry* **37**, 10945–10955.

Ebnet, K., Schulz, C.U., Meyer zu Brickwedde, M.K., Pendl, G.G. and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J Biol Chem* **275**, 27979–27988.

Ebnet, K., Aurrand-Lions, M., Kuhn, A., Kiefer, F., Butz, S., Zander, K., Meyer zu Brickwedde, M.K., Suzuki, A., Imhof, B.A. and Vestweber, D. (2003). The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity. *J Cell Sci* **116**, 3879–3891.

Fanning, A.S., Jameson, B.J., Jesiatis, L.A. and Anderson, J.M. (1998). The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* **273**, 29745–29753.

Farquhar, M.G. and Palade, G.E. (1963). Junctional complexes in various epithelia. *J Cell Biol* **17**, 375–412.

Fjellbirkeland, L., Cambier, S., Broaddus, V.C., Hill, A., Brunetta, P., Dolganov, G., Jablons, D. and Nishimura S.L. (2003). Integrin alphavbeta8-mediated activation of transforming growth factor-beta inhibits human airway epithelial proliferation in intact bronchial tissue. *Am J Pathol* **163**, 533–542.

Franke, W.W., Mueller, H., Mittnacht, S., Kapprell, H.P. and Jorcano, J.L. (1983). Significance of two desmosome plaque-associated polypeptides of molecular weights 75 000 and 83 000. *Embo J* **2**, 2211–2215.

Frisch, S. M. and Francis, H. (1994). Disruption of epithelial cell–matrix interactions induces apoptosis. *J Cell Biol* **124**, 619–626.

Fukuoka, J., Dracheva, T., Shih, J.H., Hewitt, S.M., Fujii, T., Kishor, A., Mann, F., Shilo, K., Franks, T.J., Travis, W.D. and Jen, J. (2007). Desmoglein 3 as a prognostic factor in lung cancer. *Hum Pathol* **38**, 276–283.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S (1993). Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* **123**, 1777–1788.

Furuse, M., Fujimoto, K., Sato, N., Hirase, T., Tsukita, S., and Tsukita, S. (1996). Overexpression of occludin, a tight junction-associated integral membrane protein, induces the formation of intracellular multilamellar bodies bearing tight junction-like structures. *J Cell Sci* **109**, 429–435.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. and Tsukita, S (1998a). Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* **141**, 1539–1550.

Furuse, M., Sasaki, H., Fujimoto, K. and Tsukita, S. (1998b). A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J Cell Biol* **143**, 391–401.

Furuse, M., Sasaki, H., and Tsukita, S. (1999). Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* **147**, 891–903.

Gambaletta, D., Marchetti, A., Benedetti, L., Mercurio, A.M., Sacchi, A. and Falcioni, R. (2000). Cooperative signaling between alpha(6)beta(4) integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. *J Biol Chem* **275**, 10604–10610.

Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A. and Le Meur, M. (1996). Absence of integrin alpha 6 leads to epidermolysis bullosa and neonatal death in mice. *Nat Genet* **13**, 370–373.

Ghassemifar, M.R., Sheth, B., Papenbrock, T., Leese, H.J., Houghton, F.D. and Fleming, T.P. (2002). Occludin TM4(-): an isoform of the tight junction protein present in primates lacking the fourth transmembrane domain. *J Cell Sci* **115**, 3171–3180.

Giancotti, F.G. and Ruoslahti, E. (1990). Elevated levels of the alpha 5 beta 1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell* **60**, 849–859.

Godfrey, R.W., Severs, N.J. and Jeffery, P.K. (1992). Freeze-fracture morphology and quantification of human bronchial epithelial tight junctions. *Am J Respir Cell Mol Biol* **6**, 453–458.

Gon, Y., Wood, M.R., Kiosses, W.B., Jo, E., Sanna, M.G., Chun, J. and Rosen, H. (2005). S1P3 receptor-induced reorganization of epithelial tight junctions compromises lung barrier integrity and is potentiated by TNF. *Proc Natl Acad Sci U S A* **102**, 9270–9275.

Guadagno, T.M., Ohtsubo, M., Roberts, J.M. and Assoian, R.K. (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression. *Science* **262**, 1572–1575.

Hamazaki, Y., Itoh, M., Sasaki, H., Furuse, M. and Tsukita, S. (2002). Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule. *J Biol Chem* **277**, 455–461.

Heid, H.W., Schmidt, A., Zimbelmann, R., Schäfer, S., Winter-Simanowski, S., Stumpp, S., Keith, M., Figge, U., Schnölzer, M. and Franke, W.W. (1994). Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). *Differentiation* **58**, 113–131.

Hirabayashi, S., Tajima, M., Tajima, M., Yao, I., Nishimura, W., Mori, H. and Hata, Y. (2003). JAM4, a junctional cell adhesion molecule interacting with a tight junction protein, MAGI-1. *Mol Cell Biol* **23**, 4267–4282.

Hirai, Y., Nose, A., Kobayashi, S. and Takeichi, M. (1989). Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. I. Lung epithelial morphogenesis. *Development* **105**, 263–720.

Hodivala-Dilke, K.M., DiPersio, C.M., Kreidberg, J.A. and Hynes, R.O. (1998). Novel roles for alpha3beta1 integrin as a regulator of cytoskeletal assembly and as a trans-dominant inhibitor of integrin receptor function in mouse keratinocytes. *J Cell Biol* **142**, 1357–1369.

Holtkotter, O., Nieswandt, B., Smyth, N., Müller, W., Hafner, M., Schulte, V., Krieg, T. and Eckes, B. (2002). Integrin alpha 2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen. *J Biol Chem* **277**, 10789–10794.

Huang, X., Wu, J., Zhu, W., Pytela, R. and Sheppard, D. (1998). Expression of the human integrin beta6 subunit in alveolar type II cells and bronchiolar epithelial cells reverses lung inflammation in beta6 knockout mice. *Am J Respir Cell Mol Biol* **19**, 636–642.

Huang, X., Griffiths, M., Wu, J., Farese, R.V., Jr. and Sheppard, D. (2000). Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol Cell Biol* **20**, 755–759.

Hunt, D.M., Sahota, V.K., Taylor, K., Simrak, D., Hornigold, N., Arnemann, J., Wolfe, J. and Buxton, R.S. (1999). Clustered cadherin genes: a sequence-ready contig for the desmosomal cadherin locus on human chromosome 18. *Genomics* **62**, 445–455.

Hunt, D.M., Rickman, L., Whittock, N.V., Eady, R.A., Simrak, D., Dopping-Hepenstal, P.J., Stevens, H.P., Armstrong, D.K., Hennies, H.C., Kuster, W., Hughes, A.E, Arnemann, J., Leigh, I.M., McGrath, J.A., Kelsell, D.P. and Buxton, R.S. (2001). Spectrum of dominant mutations in the desmosomal cadherin desmoglein 1, causing the skin disease striate palmoplantar keratoderma. *Eur J Hum Genet* **9**, 197–203.

Itoh, M., Morita, K. and Tsukita, S. (1999a). Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. *J Biol Chem* **274**, 5981–5986.

Itoh, M., Furuse, M., Morita K., Kubota, K., Saitou, M. and Tsukita, S. (1999b). Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J Cell Biol* **147**, 1351–1363.

Itoh, M., Sasaki, H, Furuse, M., Ozaki, H., Kita, T. and Tsukita, S. (2001). Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. *J Cell Biol* **154**, 491–497.

Jefferson, J.J., Leung, C.L. and Liem, R.K. (2004). Plakins: goliaths that link cell junctions and the cytoskeleton. *Nat Rev Mol Cell Biol* **5**, 542–553.

Kale, G., Naren, A.P., Sheth, P. and Rao, R.K. (2003). Tyrosine phosphorylation of occludin attenuates its interactions with ZO-1, ZO-2, and ZO-3. *Biochem Biophys Res Commun* **302**, 324–329.

Kasper, M., Huber, O., Grossmann, H., Rudolph, B., Trankner, C. and Muller, M. (1995). Immunocytochemical distribution of E-cadherin in normal and injured lung tissue of the rat. *Histochem Cell Biol* **104**, 383–390.

Kern, A. and Marcantonio, E.E. (1998). Role of the I-domain in collagen binding specificity and activation of the integrins alpha1beta1 and alpha2beta1. *J Cell Physiol* **176**, 634–641.

Koch, P. J., Goldschmidt, M.D., Zimbelmann, R., Troyanovsky, R. and Franke, W. W. (1992). Complexity and expression patterns of the desmosomal cadherins. *Proc Natl Acad Sci U S A* **89**, 353–357.

Koss-Harnes, D., Hyheim, B., Anton-Lamprecht, I., Gjesti, A., Jrgensen, R.S., Jahnsen, F.L., Olaisen, B., Wiche, G. and Gedde-Dahl, T. (2002). A site-specific plectin mutation causes dominant epidermolysis bullosa simplex Ogna: two identical de novo mutations. *J Invest Dermatol* **118**, 87–93.

Koukoulis, G.K., Warren, W.H., Virtanen, I. and Gould, V.E. (1997). Immunolocalization of integrins in the normal lung and in pulmonary carcinomas. *Hum Pathol* **28**, 1018–1025.

Kowalczyk, A.P., Stappenbeck, T.S., Parry, D.A., Palka, H.L., Virata, M.L., Bornslaeger, E.A., Nilles, L.A. and Green, K.J. (1994). Structure and function of desmosomal transmembrane core and plaque molecules. *Biophys Chem* **50**, 97–112.

Kowalczyk, A.P., Hatzfeld, M., Bornslaeger, E.A., Kopp, D.S., Borgwardt, J.E., Corcoran, C.M., Settler, A. and Green, K.J. (1999). The head domain of plakophilin-1 binds to desmoplakin and enhances its recruitment to desmosomes. Implications for cutaneous disease. *J Biol Chem* **274**, 18145–18148.

Kreidberg, J.A., Donovan, M.J., Goldstein, S.L., Rennke, H., Shepherd, K., Jones, R.C. and Jaenisch, R. (1996). Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development* **122**, 3537–3547.

Kubota, K., Furuse, M., Sasaki, H., Sonoda, N., Fujita, K., Nagafuchi, A. and Tsukita, S. (1999). Ca(2+)-independent cell-adhesion activity of claudins, a family of integral membrane proteins localized at tight junctions. *Curr Biol* **9**, 1035–1038.

Lapierre, L.A., Tuma, P.L., Navarrem J., Goldenring, J.R. and Anderson, J.M. (1999). VAP-33 localizes to both an intracellular vesicle population and with occludin at the tight junction. *J Cell Sci* **112**, 3723–3732.

Larjava, H., Salo, T., Haapasalmi, K., Kramer, R.H. and Heino, J. (1993). Expression of integrins and basement membrane components by wound keratinocytes. *J Clin Invest* **92**, 1425–1435.

Lee, J.O., Rieu, P., Arnaout, M.A. and Liddington,R. (1995). Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell* **80**, 631–638.

Li, Y., Fanning, A.S., Anderson, J.M. and Lavie, A. (2005). Structure of the conserved cytoplasmic C-terminal domain of occludin: identification of the ZO-1 binding surface. *J Mol Biol* **352**, 151–164.

Liang, T.W., Chiu, H.H., Gurney, A., Sidle, A., Tumas, D.B., Schow, P., Foster, J., Klassen, T., Dennis, K., DeMarco, R.A., Pham, T., Frantz, G. and Fong, S. (2002). Vascular endothelial-junctional adhesion molecule (VE-JAM)/JAM 2 interacts with T, NK, and dendritic cells through JAM 3. *J Immunol* **168**, 1618–1626.

Liu, Y., Nusrat, A., Schnell, F.J., Reaves, T.A., Walsh, S., Pochet, M. and Parkos, C,A. (2000). Human junction adhesion molecule regulates tight junction resealing in epithelia. *J Cell Sci* **113**, 2363–2374.

Lockless, S. W. and Ranganathan, R. (1999). Evolutionarily conserved pathways of energetic connectivity in protein families. *Science* **286**, 295–299.

Mankertz, J., Waller, J.S,, Hillenbrand, B., Tavalali, S., Florian, P., Schoneberg, T., Fromm, M. and Schulzke, J.D. (2002). Gene expression of the tight junction protein occludin includes differential splicing and alternative promoter usage. *Biochem Biophys Res Commun* **298**, 657–666.

Mariotti, A., Kedeshian, P.A., Dans, M., Curatola, A.M,, Gagnoux-Palacios, L. and Giancotti, F.G. (2001). EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J Cell Biol* **155**, 447–458.

Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D. and Dejana, E. (1998). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* **142**, 117–127.

Martinez-Estrada, O. M., Villa, A., Breviario, F., Orsenigo, F., Dejana, E., and Bazzoni, G. (2001). Association of junctional adhesion molecule with calcium/calmodulin-dependent serine protein kinase (CASK/LIN-2) in human epithelial caco-2 cells. *J Biol Chem* **276**, 9291–9296.

McCarthy, K.M., Francis, S.A., McCormack, J.M., Lai, J., Rogers, R.A., Skare, I.B., Lynch, R.D. and Schneeberger, E.E. (2000). Inducible expression of claudin-1-myc but not occludin-VSV-G results in aberrant tight junction strand formation in MDCK cells. *J Cell Sci* **113**, 3387–3398.

Mertens, C., Kuhn, C, Moll, R., Schwetlick, I. and Franke, W.W. (1999). Desmosomal plakophilin 2 as a differentiation marker in normal and malignant tissues. *Differentiation* **64**, 277–290.

Michelson, P.H., Tigue, M. and Jones, J.C. (2000). Human bronchial epithelial cells secrete laminin 5, express hemidesmosomal proteins, and assemble hemidesmosomes. *J Histochem Cytochem* **48**, 535–544.

Miyamoto, T., Morita, K., Takemoto, D., Takeuchi, K., Kitano, Y., Miyakawa, T., Nakayama, K., Okamura, Y., Sasaki, H., Miyachi, Y., Furuse, M. and Tsukita, S. (2005). Tight junctions in Schwann cells of peripheral myelinated axons: a lesson from claudin-19-deficient mice. *J Cell Biol* **169**, 527–538.

Mizuno, K., Suzuki, A., Hirose, T., Kitamura, K., Kutsuzawa, K., Futaki, M., Amano, Y. and Ohno, S. (2003). Self-association of PAR-3-mediated by the conserved N-terminal domain contributes to the development of epithelial tight junctions. *J Biol Chem* **278**, 31240–31250.

Morris, D.G., Huang, X., Kaminski, N., Wang, Y., Shapiro, S.D., Dolganov, G., Glick, A. and Sheppard, D. (2003). Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes MMP12-dependent emphysema. *Nature* **422**, 169–173.

Mu, D., Cambier, S., Fjellbirkeland, L., Baron, J.L., Munger, J.S., Kawakatsu, H., Sheppard, D., Broaddus, V.C. and Nishimura, S.L. (2002). The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *J Cell Biol* **157**, 493–507.

Muller, W.A. (2003). Leukocyte–endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* **24**, 327–334.

Munger, J.S., Huang, X., Kawakatsu, H., Griffiths, M.J., Dalton, S.L., Wu, J., Pittet, J.F., Kaminski, N., Garat, C., Matthay, M.A., Rifkin, D.B., Sheppard D. (1999). The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**, 319–328.

Muresan, Z., Paul, D.L. and Goodenough, D.A. (2000). Occludin 1B, a variant of the tight junction protein occludin. *Mol Biol Cell* **11**, 627–634.

Muschler, J., Lochter, A., Roskelley, C.D., Yurchenco, P. and Bissell, M.J. (1999). Division of labor among the alpha6beta4 integrin, beta1 integrins, and an E3 laminin receptor to signal morphogenesis and beta-casein expression in mammary epithelial cells. *Mol Biol Cell* **10**, 2817–2828.

Nasdala, I., Wolburg-Buchholz, K., Wolburg, H., Kuhn, A., Ebnet, K., Brachtendorf, G., Samulowitz, U., Kuster, B., Engelhardt, B., Vestweber, D. and Butz, S. (2002). A transmembrane tight junction protein selectively expressed on endothelial cells and platelets. *J Biol Chem* **277**, 16294–16303.

Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483–1487.

Neurohr, C., Nishimura, S.L. and Sheppard, D. (2006). Activation of transforming growth factor-beta by the integrin alphavbeta8 delays epithelial wound closure. *Am J Respir Cell Mol Biol* **35**, 252–259.

Niimi, T., Nagashima, K., Ward, J.M., Minoo, P., Zimonjic, D.B., Popescu, N.C. and Kimura, S. (2001). claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing. *Mol Cell Biol* **21**, 7380–7390.

Nusrat, A., Chen, J.A., Foley, C.S., Liang, T.W., Tom, J., Cromwell, M., Quan, C. and Mrsny, R.J. (2000). The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. *J Biol Chem* **275**, 29816–29822.

O'Brien, L.E., Jou, T.S., Pollack, A.L., Zhang, Q., Hansen, S.H., Yurchenco, P. and Mostov, K.E. (2001). Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat Cell Biol* **3**, 831–838.

Ohira, T., Gemmill, R.M., Ferguson, K., Kusy, S., Roche, J., Brambilla, E., Zeng, C., Baron, A., Bemis, L., Erickson, P., Wilder, E., Rustgi, A., Kitajewski, J., Gabrielson, E., Bremnes, R., Franklin, W. and Drabkin, H.A. (2003). WNT7a induces E-cadherin in lung cancer cells. *Proc Natl Acad Sci U S A* **100**, 10429–10434.

Ohno, S. (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr Opin Cell Biol* **13**, 641–648.

Ojakian, G.K. and Schwimmer, R. (1994). Regulation of epithelial cell surface polarity reversal by beta 1 integrins. *J Cell Sci* **107**, 561–576.

Ozawa, M. and Kemler, R. (1992). Molecular organization of the uvomorulin-catenin complex. *J Cell Biol* **116**, 989–996.

Paine, R., Gaposchkin, D., Kelly, C. and Wilcoxen, S.E. (1995). Regulation of cytokeratin expression in rat lung alveolar epithelial cells in vitro. *Am J Physiol Lung Cell Mol Biol* **269**, L536–L544.

Payne, A.S., Hanakawa, Y, Amagai, M. and Stanley, J.R. (2004). Desmosomes and disease: pemphigus and bullous impetigo. *Curr Opin Cell Biol* **16**, 536–543.

Peifer, M., McCrea, P.D., Green, K.J., Wieschaus, E. and Gumbiner, B.M. (1992). The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *J Cell Biol* **118**, 681–691.

Pilewski, J. M., Latoche, J.D., Arcasoy, S.M. and Albelda, S.M. (1997). Expression of integrin cell adhesion receptors during human airway epithelial repair in vivo. *Am J Physiol Lung Cell Mol Biol* **273**, L256–L263.

Pittet, J.F., Griffiths, M.J., Geiser, T., Kaminski, N., Dalton, S.L., Huang, X., Brown, L.A., Gotwals, P.J., Koteliansky, V.E., Matthay, M.A. and Sheppard, D. (2001). TGF-beta is a critical mediator of acute lung injury. *J Clin Invest* **107**, 1537–1544.

Rabinovitz, I., Tsomo, L. and Mercurio, A.M. (2004). Protein kinase C-alpha phosphorylation of specific serines in the connecting segment of the beta 4 integrin regulates the dynamics of type II hemidesmosomes. *Mol Cell Biol* **24**, 4351–4360.

Ranganathan, R. and Ross, E.M. (1997). PDZ domain proteins: scaffolds for signaling complexes. *Curr Biol* **7**, R770–R773.

Raschperger, E., Engstrom, U., Pettersson, R.F. and Fuxe, J. (2004). CLMP, a novel member of the CTX family and a new component of epithelial tight junctions. *J Biol Chem* **279**, 796–804.

Rezniczek, G.A., de Pereda, J.M., Reipert, S. and Wiche, G. (1998). Linking integrin alpha6beta4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the beta4 subunit and plectin at multiple molecular sites. *J Cell Biol* **141**, 209–225.

Roh, J.Y. and Stanley, J.R. (1995). Plakoglobin binding by human Dsg3 (pemphigus vulgaris antigen) in keratinocytes requires the cadherin-like intracytoplasmic segment. *J Invest Dermatol* **104**, 720–724.

Roh, M.H., Liu, C.J., Laurinec, S. and Margolis, B. (2002). The carboxyl terminus of zona occludens-3 binds and recruits a mammalian homologue of discs lost to tight junctions. *J Biol Chem* **277**, 27501–27509.

Ruzzi, L., Gagnoux-Palacios, L., Pinola, M., Belli, S., Meneguzz,i G., D'Alessio, M. and Zambruno, G. (1997). A homozygous mutation in the integrin alpha6 gene in junctional epidermolysis bullosa with pyloric atresia. *J Clin Invest* **99**, 2826–2831.

Saitou, M., Ando-Akatsuka, Y, Itoh, M., Furuse, M., Inazawa, J., Fujimoto, K., and Tsutkita, S. (1997). Mammalian occludin in epithelial cells: its expression and subcellular distribution. *Eur J Cell Biol* **73**, 222–231.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.D., Fromm, M., Takano, H., Noda, T. and Tsukita, S. (2000). Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* **11**, 4131–4142.

Santoso, S., Sachs, U.J., Kroll, H., Linder, M., Ruf, A., Preissner, K.T. and Chavakis, T. (2002). The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. *J Exp Med* **196**, 679–691.

Schaapveld, R.Q., Borradori, L., Geerts, D., van Leusden, M.R., Kuikman, I., Nievers, M.G., Niessen, C.M., Steenbergen, R.D., Snijders, P.J. and Sonnenberg, A. (1998). Hemidesmosome formation is initiated by the beta4 integrin subunit, requires complex formation of beta4 and HD1/plectin, and involves a direct interaction between beta4 and the bullous pemphigoid antigen 180. *J Cell Biol* **142**, 271–284.

Schlaepfer, D.D., Hanks,S.K., Hunter, T. and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372**, 786–791.

Schneeberger, E.E. (1980). Heterogeneity of tight junction morphology in extrapulmonary and intrapulmonary airways of the rat. *Anat Rec* **198**, 193–208.

Shin, K., Straight, S. and Margolis, B. (2005). PATJ regulates tight junction formation and polarity in mammalian epithelial cells. *J Cell Biol* **168**, 705–711.

Shlyonsky, V., Goolaerts, A., Van Beneden, R. and Sariban-Sohraby, S. (2005). Differentiation of epithelial Na+ channel function. An in vitro model. *J Biol Chem* **280**, 24181–24187.

Simon, D.B., Lu, Y, Choate, K.A., Velazquez, H., Al-Sabban, E., Praga, M., Casari, G., Bettinelli, A., Colussi, G., Rodriguez-Soriano, J., McCredie, D., Milford, D., Sanjad, S. and Lifton, R.P. (1999). Paracellin-1, a renal tight junction protein required for paracellular Mg2+ resorption. *Science* **285**, 103–106.

Smales, C., Ellis, M., Baumber, R., Hussain, N., Desmond, H. and Staddon, J.M. (2003). Occludin phosphorylation: identification of an occludin kinase in brain and cell extracts as CK2. *FEBS Lett* **545**, 161–166.

Smythe, W.R., Williams, J.P., Wheelcock, M.J., Johnson, K.R., Kaiser, L.R. and Albelda, S.M. (1999). Cadherin and catenin expression in normal human bronchial epithelium and non-small cell lung cancer. *Lung Cancer* **24**, 157–168.

Sorokin, L., Sonnenberg, A., Aumailley, M., Timpl, R. and Ekblom, P. (1990). Recognition of the laminin E8 cell-binding site by an integrin possessing the alpha 6 subunit is essential for epithelial polarization in developing kidney tubules. *J Cell Biol* **111**, 1265–1273.

Stepp, M.A., Spurr-Michaud, S., Tisdale, A., Elwell, J. and Gipson, I.K. (1990). Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc Natl Acad Sci U S A* **87**, 8970–8974.

Sterk, L.M., Geuijen, C.A., Oomen, L.C., Calafat, J., Janssem, H. and Sonnenberg, A. (2000). The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin alpha6beta4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol* **149**, 969–982.

Straight, S.W., Shin, K., Fogg, V.C., Fan, S., Liu, C.J., Roh, M. and Margolis, B. (2004). Loss of PALS1 expression leads to tight junction and polarity defects. *Mol Biol Cell* **15**, 1981–1990.

Streuli, C.H., Bailey, N. and Bissell, M.J. (1991). Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell–cell interaction and morphological polarity. *J Cell Biol* **115**, 1383–1395.

Stuart, R. O. and Nigam, S.K. (1995). Regulated assembly of tight junctions by protein kinase C. *Proc Natl Acad Sci U S A* **92**, 6072–6076.

Takabayshi, K., Corr, M., Hayashi, T., Redecke, V., Beck, L., Guiney, D., Sheppard, D. and Raz, E. (2006). Induction of a homeostatic circuit in lung tissue by microbial compounds. *Immunity* **24**, 475–487.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451–1455.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* **7**, 619–627.

Thomas, G.J., Poomsawat, S., Lewis, M.P., Hart, I.R., Speight, P.M. and Marshall, J.E. (2001). alpha v beta 6 Integrin upregulates matrix metalloproteinase 9 and promotes migration of normal oral keratinocytes. *J Invest Dermatol* **116**, 898–904.

Tournier, J.M., Goldstein, G.A., Hall, D.E., Damsky, C.H. and Basbaum, C.B. (1992). Extracellular matrix proteins regulate morphologic and biochemical properties of tracheal gland serous cells through integrins. *Am J Respir Cell Mol Biol* **6**, 461–471.

Troyanovsky, S.M. and Leube R.E. (1998). Molecular dissection of desmosomal assembly and intermediate filament anchorage. *Subcell Biochem* **31**, 263–289.

Troyanovsky, S.M., Troyanovsky, R.B., Eshkind, L.G., Krutovskikh, V.A., Leube, R.E. and Franke, W.W. (1994a). Identification of the plakoglobin-binding domain in desmoglein and its role in plaque assembly and intermediate filament anchorage. *J Cell Biol* **127**, 151–160.

Troyanovsky, S.M., Troyanovsky, R.B., Eshkind, L.G., Leube, R.E. and Franke, W.W. (1994b). Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation. *Proc Natl Acad Sci U S A* **91**, 10790–10794.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* **2**, 285–293.

Umeda, K., Ikenouchi, J., Katahira-Tayama, S., Furuse, K., Sasaki, H., Nakayama, M., Matsui, T., Tsukita, S., Furuse, M. and Tsukita, S. (2006). ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* **126**, 741–754.

van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C.M. and Sonnenberg, A. (1996). Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. *Nat Genet* **13**, 366–369.

Van Itallie, C.M. and Anderson, J.M. (2004). The molecular physiology of tight junction pores. *Physiology (Bethesda)* **19**, 331–338.

Varner, J.A., Emerson, D.A. and Juliano, R.L. (1995). Integrin alpha 5 beta 1 expression negatively regulates cell growth: reversal by attachment to fibronectin. *Mol Biol Cell* **6**, 725–740.

Walters, R.W., Grunst, T., Bergelson, J.M., Finberg, R.W., Welsh, M.J. and Zabner, J. (1999). Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. *J Biol Chem* **274**, 10219–10226.

Wang, F., Daugherty, B., Keise, L.L., Wei, Z., Foley, J.P., Savani, R.C. and Koval, M. (2003). Heterogeneity of claudin expression by alveolar epithelial cells. *Am J Respir Cell Mol Biol* **29**, 62–70.

Wary, K.K., Mariotti, A., Zurzolo, C. and Giancotti, F.G. (1998). A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* **94**, 625–634.

Watt, F.M. (2002). Role of integrins in regulating epidermal adhesion, growth and differentiation. *Embo J* **21**, 3919–3926.

Weinacker, A., Ferrando, R., Elliott, M., Hogg, J., Balmes, J. and Sheppard, D. (1995). Distribution of integrins alpha v beta 6 and alpha 9 beta 1 and their known ligands, fibronectin and tenascin, in human airways. *Am J Respir Cell Mol Biol* **12**, 547–556.

Weitzman, J.B., Chen, A. and Hemler, M.E. (1995). Investigation of the role of beta 1 integrins in cell–cell adhesion. *J Cell Sci* **108**, 3635–3644.

West, M.R., Ferguson, D.J., Hart, V.J., Sanjar, S. and Man, Y. (2002). Maintenance of the epithelial barrier in a bronchial epithelial cell line is dependent on functional E-cadherin local to the tight junctions. *Cell Commun Adhes* **9**, 29–44.

Wilcox, E.R., Burton, Q.L., Naz, S., Riazuddin, S., Smith, T.N., Ploplis, B., Belyantseva, I., Ben-Yosef, T., Liburd, N.A., Morell, R.J., Kachar, B., Wu, D.K., Griffith, A.J., Riazuddin, S. and Friedman, T.B. (2001). Mutations in the gene encoding tight junction claudin-14 cause autosomal recessive deafness DFNB29. *Cell* **104**,165–172.

Williams, L.A., Martin-Padura, I., Dejana, E., Hogg, N. and Simmons, D.L. (1999). Identification and characterisation of human Junctional Adhesion Molecule (JAM). *Mol Immunol* **36**, 1175–1188.

Winn, R.A., Bremnes, R.M., Bemis, L., Franklin, W.A., Miller, Y.E., Cool, C. and Heasley, L.E. (2002). gamma-Catenin expression is reduced or absent in a subset of human lung cancers and re-expression inhibits transformed cell growth. *Oncogene* **21**, 7497–7506.

Winter, M.C., Shasby, S.S,, Ries, D.R. and Shasby, D.M. (2006). PAR2 activation interrupts E-cadherin adhesion and compromises the airway epithelial barrier: protective effect of beta-agonists. *Am J Physiol Lung Cell Mol Physiol* **291**, L628–L635.

Wittchen, E.S., Haskins, J. and Stevenson, B.R. (1999). Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J Biol Chem* **274**, 35179–35185.

Wittchen, E.S., Haskins, J. and Stevenson, B.R. (2000). Exogenous expression of the amino-terminal half of the tight junction protein ZO-3 perturbs junctional complex assembly. *J Cell Biol* **151**, 825–836.

Xiong, J.P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S.L. and Arnaout, M.A. (2002). Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science* **296**, 151–155.

Xue, H., Atakilit, A., Zhu, W., Li, X., Ramos, D.M. and Pytela, R. (2001). Role of the alpha(v)beta6 integrin in human oral squamous cell carcinoma growth in vivo and in vitro. *Biochem Biophys Res Commun* **288**, 610–618.

Yamada, T., Endo, R., Tsukagoshi, K., Fujita, S., Honda, K., Kinoshita, M., Hasebe, T. and Hirohashi, S. (1996). Aberrant expression of a hemidesmosomal protein, bullous pemphigoid antigen 2, in human squamous cell carcinoma. *Lab Invest* **75**, 589–600.

Young, G.D., Winokur, T.S., Cerfolio, R.J., Van Tine, B.A., Chow, L.T., Okoh, V. and Garver, R.I., Jr. (2002). Differential expression and biodistribution of cytokeratin 18 and desmoplakins in non-small cell lung carcinoma subtypes. *Lung Cancer* **36**, 133–141.

Young, S.L., Chang, L.Y. and Erickson, H.P. (1994). Tenascin-C in rat lung: distribution, ontogeny and role in branching morphogenesis. *Dev Biol* **161**, 615–625.

Yuan, B., Li, C., Kimura, S., Engelhardt, R.T., Smith, B.R. and Minoo, P. (2000). Inhibition of distal lung morphogenesis in Nkx2.1(-/-) embryos. *Dev Dyn* **217**, 180–190.

3

The Epithelium as a Target

Louise E. Donnelly
National Heart and Lung Institute, Imperial College London, UK

3.1 Introduction

The epithelial cells that line the airway consist of several different types including ciliated columnar cells, basal cells and secretory/goblet cells. The distribution pattern of the different types of epithelial cells changes from the bronchi down to the alveoli. In the bronchi, the airway epithelium consists of ciliated epithelial cells, goblet cells and a few Clara cells, whereas in the small airway the cells are less columnar and more cuboidal with increased numbers of Clara cells. This array of cell types forms the airway epithelium and is the first line of defence against airborne agents including allergens and irritants. Furthermore, these cells not only form a physical barrier between the airway lumen and the interstitium but they also have the capacity to exhibit many pro- and anti-inflammatory features and may actively participate in the inflammatory processes in the lung. Therefore, any disruption of the normal functioning of the airway epithelium could contribute to and/or exacerbate disease processes in the lung. Exactly how the airway epithelium is modified or damaged in inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease is currently under investigation. This chapter will describe the mechanisms of damage to the airway epithelium and how these contribute to disease pathophysiology.

3.2 Asthma

Shedding of the airway epithelium is a common histological feature observed in biopsies obtained from asthmatic patients (Jeffery, 2004). Although this desquamation is variable in mild asthmatics and may be due to sampling techniques (Ordonez *et al*., 2000), the presence of epithelial cells in induced sputum from asthmatic patients (Creola bodies) and the correlation of epithelial shedding with airway hyperresponsiveness in more severe disease (Jeffery *et al*., 1989; Tateishi *et al*., 1996) would suggest that damage to the epithelium contributes to the underlying pathophysiology of asthma.

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

The mechanism of this heightened responsiveness associated with epithelial shedding is unclear, but compromised integrity of the airway barrier may increase the sensitivity of the airway to various stimuli including neuropeptides such as substance P (Joos *et al*., 1994). Similarly, alteration of the epithelium could increase permeability of the airway mucosa and exacerbate exudation of plasma into the airway (Goldie and Pedersen, 1995). In addition, the presence of Th2 lymphocytes in the airway of these patients (Robinson *et al*., 1992, 1993) would suggest a predominance of Th2 cytokines, including interleukin (IL)-4 and IL-13 (Wong *et al*., 2001), which have profound effects on the airway epithelium causing these cells to release chemotactic agents including eotaxin (CXCL11) (Li *et al*., 1999), and to stimulate mucin production (Wu *et al*., 1990).

3.2.1 Allergen exposure

Asthma exacerbations are triggered by a number of stimuli including viral infections, cold air, pollution and exercise, but allergen exposure is one of the most prevalent factors associated with the development of asthma, and with the frequency of subsequent exacerbations. The airway epithelium is the first target for inhaled allergens. These cells cannot process allergen but are capable of trafficking these particles such that the allergen translocates from the airway lumen to the submucosa where it can come into contact with underlying antigen presenting cells, including dendritic cells (Mattoli, 2001). In asthma, where the epithelial layers have become denuded, these particles may interact directly and stimulate the mucosa and possibly the airway smooth muscle. Some of the most common allergens are derived from house dust mite, *Dermatophagoides pteronyssinus*, and include the proteases designated (Der p) proteins. A number of studies have examined the effects of these Der p proteins on airway epithelium and demonstrated disruption of the tight junctions between the epithelial cells via their innate protease activity (Kauffman *et al*., 2006; Page *et al*., 2006). Thus, allergen exposure to the epithelium can contribute to epithelial shedding (Herbert *et al*., 1995) and hence facilitate trafficking of the allergens (Wan *et al*., 1999). Indeed, avoidance of the allergen can reduce epithelial cell numbers in induced sputum from asthmatic children (Piacentini *et al*., 1998) further supporting the hypothesis that damage to the epithelium in asthma is driven by allergen challenge. It is, therefore, conceivable that allergen could stimulate directly the airway smooth muscle. Indeed, it has been demonstrated that exposure of airway smooth muscle to Der p1 will enhance smooth muscle constrictor responses and lead to activation of mitogen-activated protein (MAP) kinase pathways which in turn could regulate inflammatory mediator production (Grunstein *et al*., 2005).

Allergen-driven epithelial responses include the production of IL-8 (CXCL8) and IL-6 following stimulation with Der p1 and Der p5 proteins (Adam *et al*., 2006; Asokananthan *et al*., 2002; Kauffman *et al*., 2006). These allergen-derived proteases exert their effects on the airway epithelium via activation of the protease-activated receptors (PAR), in particular PAR-2 (Asokananthan *et al*., 2002). Similar responses have been observed when airway epithelial cells were exposed to other allergens derived from German cockroach extracts that again mediate the release of IL-8 (Hong *et al*., 2004; Page *et al*., 2003). Activation of the epithelial cell layer with these antigens is also dependent upon activation of PAR-2 (Hong *et al*., 2004; Page *et al*., 2003); however, PAR-independent pathways leading to cytokine production also exist, but the relative importance of each pathway is yet to be evaluated (Adam *et al*., 2006; Kauffman *et al*., 2006). Stimulation of the airway epithelium with allergen could contribute to the increased inflammatory cell influx associated with the

underlying chronic inflammation observed in asthma. For example, Der p induces the release of CCL17 (thymus and activation regulated chemokine – TARC) from epithelial cells, the levels of which can be further enhanced when co-stimulated with IL-4 and transforming growth factor (TGF)β (Heijink *et al*., 2006). CCL17 is a ligand for the chemokine receptor CCR4 which is expressed by T-lymphocytes, therefore allergen stimulation of the epithelium could drive the inflammatory process in asthma directly. Similarly, antigen stimulation of the epithelium leads to the production of the potent neutrophil chemoattractant, IL-8 (Adam *et al*., 2006; Page *et al*., 2003), thereby regulating neutrophil trafficking into the airway.

3.2.2 Inflammatory cells

The inflammatory influx observed in the lungs of asthmatic subjects is characterized by increased numbers of eosinophils, mast cells, and T-lymphocytes following allergen challenge (Bousquet *et al*., 2000). Indeed, the numbers of eosinophils in bronchoalveolar lavage (BAL) correlates with the numbers of epithelial cells present, suggesting an association between eosinophilic inflammation and epithelial damage (Oddera *et al*., 1996).

Following allergen challenge, eosinophils will migrate towards the airway lumen and are located at the sites of epithelial damage (Erjefalt *et al*., 1997). Although such studies cannot exclude the possibility that the eosinophil may be participating in the repair process, the observation that co-culture of eosinophils with bronchial epithelial cells leads to CD18-dependent degranulation of the eosinophil and the release of cytotoxic mediators including eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil-derived neurotoxin (Takafuji *et al*., 1996) would suggest that eosinophils are involved in damage of the airway epithelium in asthma. Furthermore, the presence of increased levels of IL-5 in the airway of asthmatic subjects promotes the adhesion of eosinophils to the airway epithelium via upregulation of CD18 and α4 integrins (Sanmugalingham *et al*., 2000). Interestingly, activation of co-cultures of eosinophils and epithelial cells with Der p1 antigens increases epithelial expression of ICAM-1 and a concomitant induction of CD18 and ICAM-1 by eosinophils, thus mediating the adhesion of these cells leading to production of IL-1β, IL-6, IL-10, tumour necrosis factor (TNF)-α and granulocyte macrophage-colony stimulating factor (GM-CSF) via NF-κB, AP-1 and p38 MAP kinase dependent mechanisms (Wong *et al*., 2006).

Such activation of eosinophils can drive both apoptosis and necrosis of human primary airway epithelial cells *in vitro* but despite the fact that other cytotoxic molecules are released following degranulation of the eosinophil, apoptosis of the airway epithelium is thought to be mediated mainly via the production of TNF-α from these cells (Trautmann *et al*., 2002). This then leads to the possibility that other inflammatory cells in the asthmatic airway such as macrophages and mast cells could contribute to epithelial cell death via the production of TNF-α. Furthermore, this effect is potentiated by the presence of interferon (IFN)-γ and has led to the observation that T-cells and eosinophils co-operate to induce epithelial damage (Trautmann *et al*., 2002). Despite such compelling evidence, whether eosinophils are responsible for epithelial damage remains controversial since murine models of eosinophilic inflammation do not show any alteration in epithelial fragility (Blyth *et al*., 1996).

Other inflammatory cells that will contribute to the damaged epithelium include neutrophils. In children undergoing acute exacerbations, epithelial damage is associated with an increase in IL-8 but not with an enhanced eosinophilic signal (Yoshihara *et al*., 2006).

Moreover, in severe asthma there is a marked increase in the presence of neutrophils in the airway (Wenzel, 2003) suggesting an altered pathophysiology compared to less severe asthma. Neutrophils contain azurophilic granules that release proteases including neutrophil elastase, cathepsin G and proteinase-3 that can damage the airway epithelium. Moreover, the persistence of neutrophils may be enhanced since the airway epithelium is not capable of phagocytosis of apoptotic neutrophils, which contrasts with its ability to remove apoptotic eosinophils (Sexton *et al*., 2004). The effects of neutrophils on the airway epithelium may be potentiated during viral induced exacerbations. Infection of the airway epithelium with respiratory syncytial virus (RSV) enhances neutrophil adhesion and increased damage and epithelial shedding (Wang *et al*., 1998). The mechanisms for these interactions include the RSV-induced upregulation of neutrophil chemoattractants in the epithelium, together with increased expression of adhesion molecules, including ICAM-1 on the airway epithelium and CD18 on the neutrophil, leading to activation of neutrophils and release of cytotoxic mediators including proteases (Wang and Forsyth, 2000).

3.2.3 Glucocorticosteroids

Glucocorticosteroids are the mainstay of anti-inflammatory therapy in asthma. These drugs can reduce the inflammation in the airway by promoting apoptosis of eosinophils and T-lymphocytes thus reducing the inflammatory load (Meagher *et al*., 1996; Melis *et al*., 2002; O'Sullivan *et al*., 2004). However, while controversial, it has been reported that glucocorticosteroids also induce apoptosis in airway epithelial cells (Dorscheid *et al*., 2001; White and Dorscheid, 2002), raising the prospect that this treatment could be responsible for the persistence of epithelial damage observed in patients with chronic asthma. Recently, combination therapies of β_2-adrenoceptor agonists and glucocorticosteroids have been shown to be more effective at reducing the inflammatory load in moderate asthma when compared with inhaled steroids alone (Ankerst, 2005; Currie *et al*., 2005). This may be of benefit to the airway epithelium as the apoptotic effect of glucocorticosteroids can be reduced by co-administration of β_2-adrenoceptor agonists (Tse *et al*., 2003), thus the combination therapies currently available may prevent epithelial damage in these patients.

3.3 Alteration in epithelial cell type distribution

A clear feature of asthma is increased expression of mucin reflecting changes in goblet cells or submucosal gland function (Rogers, 2002). Goblet cell hyperplasia is observed in the large airway in asthmatic subjects (Shimura *et al*., 1996), with up to threefold more cells in bronchial biopsies of subjects with mild asthma compared with control subjects (Ordonez *et al*., 2001). Whether this increase in goblet cell number reflects basal epithelial cell differentiation or goblet cell division remains to be elucidated. However, in most cases the source of goblet cells is via differentiation of non-granulated epithelial cells (Rogers, 2002) but whether this occurs during disease pathology is not known. Currently, there is little evidence in asthma that this increase in goblet cell number reflects a loss in the numbers of progenitor cells such as the basal cells, surface epithelial serous cells or Clara cells.

An alteration in Clara cell number could alter the host response of the airway as these cells are responsible for the production of a number of anti-inflammatory molecules including lactoferrin and secretory leukocyte protease inhibitor (Rogers, 2002).

3.4 Overview of epithelial damage in asthma

There are clearly a number of factors that contribute to epithelial dysfunction in asthma. Whether allergens, pollutants or infectious agents are the initiating factor for epithelial damage in asthmatic subjects is unknown, but they certainly can work alone or in concert to promote epithelial fragility (Figure 3.1). This can, in turn, promote leukocyte influx and mediate the chronic inflammation observed in the asthmatic lung (Figure 3.1), which can also contribute to epithelial damage. This cycle of epithelial damage and inflammation should be ameliorated by anti-inflammatory agents; however, since glucocorticoids may also promote epithelial cell apoptosis, it appears that alternative anti-inflammatory strategies are warranted to maintain epithelial integrity in the asthmatic airway.

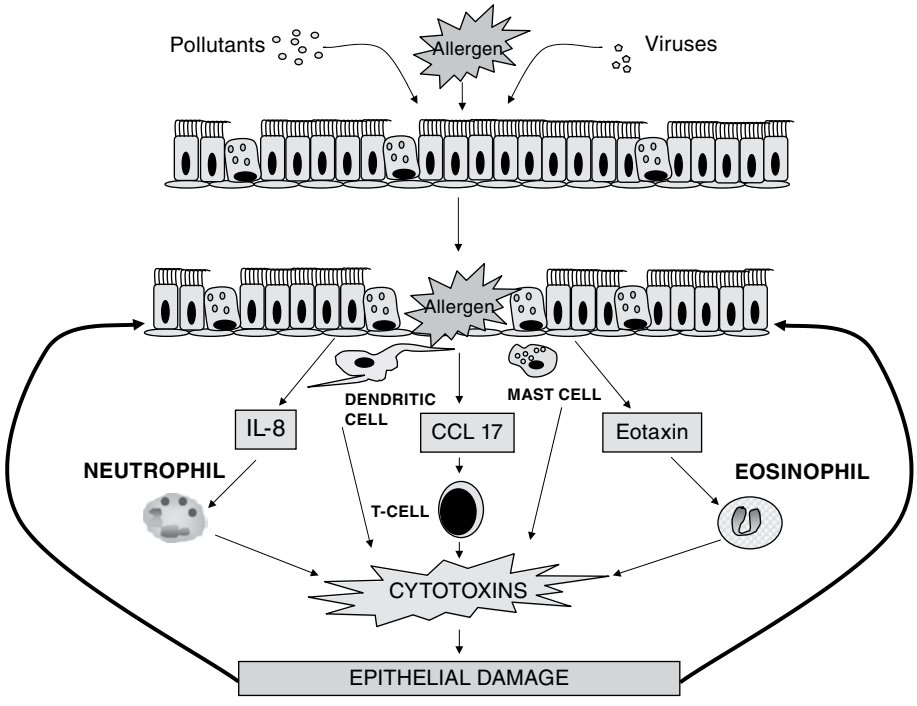


Figure 3.1 Putative mechanisms of epithelial damage in asthma. Allergens, pollutants or infectious agents such as viral particles can adhere to the airway epithelium and mediate permeability of tight junctions and stimulate the release of inflammatory mediators leading to epithelial shedding and inflammatory cell recruitment. Various inflammatory subtypes are recruited into the intraepithelial spaces and stimulate the epithelium, enhancing inflammatory mediator production. Subsequent activation of the inflammatory cells leads to the release to cytotoxic substances that mediate the apoptosis or necrosis of the airway epithelium, thus contributing to the cycle of epithelial damage and inflammation observed in asthma

3.5 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is largely irreversible (GOLD, 2001) and is associated with an underlying inflammation consisting predominantly of macrophages, T-lymphocytes and neutrophils (Donnelly and Barnes, 2006). The major cause of COPD is cigarette smoking but only approximately 15–20% of smokers will develop this disease (Lindberg *et al*., 2006). Unlike asthma, the airway epithelium is not denuded in COPD; however, there is marked goblet cell hyperplasia and squamous metaplasia (Jeffery, 2004). In addition, the amount of mucin stored within these cells is also increased and shows a correlation with airflow obstruction (Innes *et al*., 2006).

Airway epithelial cells from COPD patients have been shown to exhibit differential patterns of gene expression when compared with cells from smokers without COPD and cells from nonsmokers (Pierrou *et al*., 2006). These differences were mainly reflected in genes regulating oxidant stress responses, and these responses were reproduced when cells were cultured *in vitro* and exposed to cigarette smoke extract (CSE) (Pierrou *et al*., 2006). Studies examining airway epithelial cells derived from bronchial brushings from these patients have also shown differences in inflammatory mediator expression when compared with cells obtained from asymptomatic smokers and from nonsmokers. For example, transforming growth factor (TGF)β expression is increased in small airway epithelial cells and correlates with obstruction and smoking history (Takizawa *et al*., 2001). Culture of airway epithelial cells from these patients also shows an enhanced response to inflammatory stimuli. For example, stimulation of these cells with TNFα leads to increased release of IL-8 and IL-6 by cells from COPD patients compared with smokers with normal lung function (Patel *et al*., 2003). Similarly, exposure of cells to a combination of TNFα and IFNγ also demonstrated increased expression of IL-8 and a second neutrophil chemoattractant CXCL1 (growth-related oncogene-α – GROα) (Schulz *et al*., 2004). Such observations have led to studies examining the effects of the major stimulus in COPD, namely cigarette smoke, usually in the form of CSE, on inflammatory gene expression and mediator release by airway epithelial cells.

3.6 Effect of cigarette smoke

Exposure of bronchial epithelial cells to CSE increases release of IL-8 (Glader *et al*., 2006; Mio *et al*., 1997). By contrast, IL-8 release is inhibited following exposure of type II alveolar epithelial cells to CSE (Witherden *et al*., 2004), suggesting differential responses of pulmonary epithelial cells could regulate the inflammatory influx observed in COPD. IL-8 is a neutrophil chemoattractant and a marked neutrophilia is observed in the large airway of these patients and is reflected by increased neutrophil numbers in the induced sputum of these patients when compared with smokers and nonsmokers (Keatings *et al*., 1996; Traves *et al*., 2002). This neutrophilia is not observed in bronchoalveolar lavage samples which are thought to reflect the alveolar airspaces (Traves *et al*., 2002). Therefore, release of IL-8 from the bronchial epithelium following stimulation with cigarette smoke could be responsible for the neutrophil influx in the large airway but not in the alveoli.

CSE also affects other aspects of epithelial cell function including stimulation of proliferation at low concentrations via the activation of ERK MAP kinases (Luppi *et al*., 2005);

however, increasing concentrations of CSE led to inhibition of this response and activation of the p38 MAP kinase pathway (Luppi *et al*., 2005) with inhibition of the release of TGF-β and fibronectin, thereby inhibiting the epithelial repair process (Wang *et al*., 2001). However, the relevance of the concentrations of CSE to exposure of the airway epithelium to cigarette smoke in smokers is not known. In an attempt to address this problem, other workers have developed methodologies to expose airway epithelial cells to cigarette smoke directly.

Exposure of epithelial cells to cigarette smoke leads to activation of p38 MAP kinase and the transcription factor NF-κB (Beisswenger *et al*., 2004) suggesting that smoke can directly stimulate cells to produce inflammatory mediators. Additionally, exposure of cells to cigarette smoke increased the permeability of the epithelial cell layer but this effect was increased in cells from COPD patients compared with cells with normal lung function (Rusznak *et al*., 2000). Moreover, expression of IL-1β and sICAM-1 by these cells was also increased and associated with a concomitant decrease in intracellular glutathione levels, suggesting that an oxidant stress mediates this response (Rusznak *et al*., 2000).

Cigarette smoke contains more than 4700 components therefore it is difficult to determine exactly which component is responsible for the observed effects of cigarette smoke. Nevertheless, many studies have demonstrated that exposure of the epithelium to CSE induces an oxidative stress (Bowler *et al*., 2004; Marwick *et al*., 2002; Moodie *et al*., 2004; Rahman and MacNee 1999). Oxidants stimulate the airway epithelium via activation of NF-κB leading to the expression of many inflammatory genes including IL-8 and matrix metalloproteinase (MMP)-9 (Hozumi *et al*., 2001; Tomita *et al*., 2003); furthermore oxidant exposure of cells leads to inactivation and down regulation of histone deacetylase (HDAC)-2 leading to glucocorticosteroid insensitivity (Ito *et al*., 2001, 2006; Tomita *et al*., 2003). This may be a particularly important mechanism in COPD as the underlying inflammatory response is steroid-resistant (Barnes, 2000b; Barnes *et al*., 2003; Culpitt *et al*., 1999; Ito *et al*., 2001) and could be related to cigarette smoke exposure. Not only can cigarette smoke mediate inflammatory responses in the airway epithelium, it can also potentiate the effect of other damaging agents. For example, cigarette smoke potentiates the effect of the house dust mite allergen, Der p1, to increase the permeability of the epithelial layer and the release of inflammatory mediators including IL-8, IL-1β and sICAM-1 (Rusznak *et al*., 2001, 1999).

3.7 Other causative factors

Cigarette smoke is not the only causative agent in the development of COPD. Burning of biomass fuels and air pollutants have also been implicated in the aetiology of airflow obstruction and chronic bronchitis (Ekici *et al*., 2005; Perez-Padilla *et al*., 1996), although the exact mechanisms leading to the pathophysiology of the obstruction remain to be elucidated. Ambient air pollution particles are also respirable and will target the epithelium.

3.7.1 Pollution

Experimental models of *in vitro* cell culture or explanted trachea have shown that the airway epithelium is capable of taking up diesel exhaust particles by endocytic mechanisms (Boland *et al*., 1999). Indeed, such particles can translocate through the epithelial layer into the underlying submucosa and induce fibrosis in the airway wall (Churg and Wright, 2002).

These particles are not inert and generate an oxidative stress (Tao *et al*., 2003) together with activation of NF-κB causing inflammatory gene transcription and potentiating the inflammatory response leading to release of IL-1β and IL-8 (Boland *et al*., 1999; Churg and Wright, 2002). Other atmospheric pollutants will also impact upon the responses of the airway epithelium. For example, ozone and nitrogen dioxide will induce the release of a variety of inflammatory cytokines from the airway epithelium and increase epithelial permeability (Bayram *et al*., 2002; Bosson *et al*., 2003). Indeed, it has been proposed that the release of Clara cell protein, CC16, can be used as a marker of ozone-induced lung injury and can be measured in serum in the absence of other markers of epithelial damage such as albumin in the bronchoalveolar lavage fluid (Blomberg *et al*., 2003).

3.7.2 Infection

The incidence and prevalence of bacterial infections are associated with an accelerated decline in lung function in COPD (Donaldson *et al*., 2002). The major bacterial pathogens in COPD are *Haemophilus influenzae, Streptoccocus pneumoniae* and *Moxarella catarrhalis*, though exactly why COPD patients are more prone to these infections is unknown. In COPD patients colonized with *H. influenzae* there is a neutrophil infiltration; however, defensins released by neutrophils from these patients were incapable of killing this bacteria (Gorter *et al*., 1998). Indeed, adhesion of *H. influenzae* to the epithelial cell surface was enhanced by the presence of neutrophil defensins (Gorter *et al*., 1998). Moreover, exposure of the epithelium to *H. influenzae*-derived proteins and the bacteria itself can stimulate the cells to produce IL-8 via activation of the MAP kinase pathways (Wang *et al*., 2003) an effect that can be potentiated in the presence of TNF-α via activation of NF-κB (Watanabe *et al*., 2004). Similarly, *H. influenzae* and rhinovirus infection can enhance the expression of the neutrophil chemoattractants IL-8, CXCL1 and CXCL5 and upregulate the expression of ICAM-1 and toll-like receptor (TLR)-3 (Sajjan *et al*., 2006) which could increase the susceptibility of the epithelium to enhanced adhesion and further activation.

3.8 Alveolar epithelial cell apoptosis – emphysema

A key feature of COPD is the development of emphysema (Barnes, 2000a). Emphysema is the destruction of the alveolar airspaces, a key component of which is the apoptosis or necrosis of alveolar epithelial cells. The turnover of the alveolar epithelium in patients with emphysema is not well understood; however, biopsy studies have revealed there are enhanced proliferative and apoptotic processes occurring in the alveolar epithelium from emphysematous patients compared with tissue from asymptomatic smokers and nonsmokers (Yokohori *et al*., 2004). Interestingly, increased levels of markers of epithelial cell apoptosis persist following smoking cessation in patients with COPD suggesting that this effect may not be related directly to smoking (Hodge *et al*., 2005). Exactly why the epithelium from emphysematous patients shows increased turnover is not clear; however, mediators of epithelial apoptosis are under investigation. Blockade of vascular endothelial growth factor (VEGF) receptors leads to alveolar cell apoptosis and the development of emphysema in rat models (Kasahara *et al*., 2000). Subsequently, it was proposed that oxidative stress could reduce the levels of VEGF promoting apoptosis of the epithelium (Tuder *et al*., 2003). Cigarette smoke could therefore contribute to alveolar apoptosis via increased oxidative stress; however, it

is also possible that cigarette smoke induces necrosis of alveolar epithelial cells directly via inhibition of caspases and thus contributes to the development of emphysema (Wickenden *et al*., 2003).

Other mechanisms of epithelial cell apoptosis leading the development of emphysema have also been proposed. There is an increase in the numbers of $CD8^+$ T-lymphocytes in the lungs of patients with COPD (Saetta *et al*., 1999). These cytotoxic T-cells cells contain granzyme and perforins, which together with TNF-α can induce epithelial cell death (Shinbori *et al*., 2004). The $CD8^+$ T-cells in COPD lung express high levels of CXCR3 (Saetta *et al*., 2002). The three ligands for this receptor, CXCL9, CXCL10 and CXCL11 are all released by airway epithelial cells following stimulation with IFN-γ (Cole *et al*., 1998; Mohan *et al*., 2002; Sauty *et al*., 1999) and can be potentiated by TNF-α (Mohan *et al*., 2002; Sauty *et al*., 1999). The concentrations of these cytokines are increased in COPD due to the presence of inflammatory cells including macrophages and T-lymphocytes, thereby perpetuating an inflammatory cycle leading to the destruction of the lung tissue (Figure 3.2).

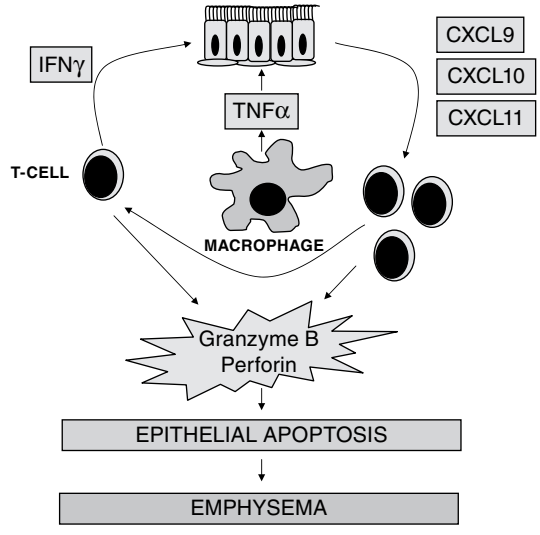


Figure 3.2 Airway epithelial cell–T-cell interactions in the development of emphysema. Airway epithelial cells will produce CXCR3 chemokines, CXCL9, CXCL10 and CXCL11 upon stimulation with T-cell-derived IFNγ. This effect can be potentiated by TNFα released by activated macrophages. Recruitment of $CD8^+$ cytotoxic T-cells will lead to the release of cytotoxic molecules such as granzyme B and perforin which promote apoptosis of the alveolar epithelium and ultimately emphysema

3.9 Overview of epithelial damage in COPD

There are a number of factors that contribute to epithelial dysfunction in COPD. By far the most important risk factor for the development of COPD is cigarette smoking. The oxidant stress associated with cigarette smoke will stimulate the epithelium to produce

inflammatory mediators and upregulate adhesion molecules which facilitate the binding of bacterial pathogens to the cell surfaces (Figure 3.3). These factors mediate the inflammatory response by recruitment and activation of macrophages, neutrophils and T-cells which, in turn, release pro-inflammatory mediators and proteases that alter epithelial permeability and contribute to the characteristic desquamation of the airway epithelium (Figure 3.3).

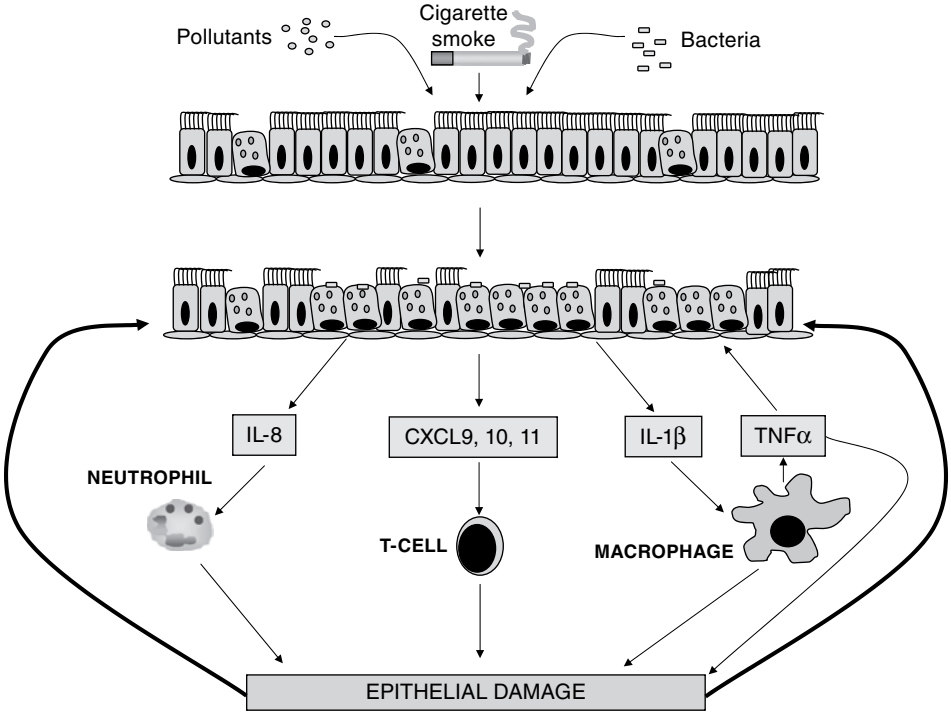


Figure 3.3 Putative mechanisms of epithelial damage in COPD. Cigarette smoke, pollutants and bacteria can interact with the airway epithelium and mediate permeability of tight junctions and stimulate the release of inflammatory mediators leading to desquamation of the surface with loss of ciliated epithelium. There is an upregulation of adhesion molecules leading to enhanced bacterial adhesion and further activation of the epithelium. This enhances inflammatory cell recruitment including neutrophils and macrophages which produce damaging proteases including neutrophil elastase and MMPs. In addition, macrophages will also contribute to the release of TNFα which can mediate apoptosis of the epithelial cells. Cytotoxic substances will also be released by invading T-cells and together mediate damage to the epithelium

3.10 Damage to the epithelium in other diseases

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR), an epithelial chloride channel that regulates periciliary lining fluid ion concentrations. Failure of this channel is associated with mucous plugging, impaired

mucillary clearance and persistent infection of the airway most notably with *Pseudomonas aeruginosa* (Chmiel and Davis, 2003). However, the lack of functioning CFTR is not the only difference in the airway epithelial cells lining the lungs of these patients that may contribute to the disease pathology. Airway epithelial cells from CF patients express increased levels of IL-8 compared with cells derived from healthy controls (Sajjan *et al*., 2004). As neutrophil accumulation in the lungs of these patients is a common histopathological feature of CF, it would appear that activation of the airway epithelium could mediate this neutrophilia. Adherence of neutrophils is also enhanced in airway epithelial cells from CF patients leading to a heightened inflammatory response with further increases in the release of IL-6 and IL-8 (Tabary *et al*., 2006). Furthermore, the adherence of neutrophils to the epithelial surface appears to mediate damage via the release of proteases (Venaille *et al*., 1998). Adhesion of *P. aeruginosa* also stimulates the airway epithelium leading to the the release of IL-8 from these cells (Delgado *et al*., 2006). However, adhesion only occurs during repair processes and requires expression of asialo ganglioside M1 (de Bentzmann *et al*., 1996a, 1996b).

Obliterative bronchiolitis can occur during rejection of lung transplant patients and is characterized by epithelial damage. This is an irreversible process that is not understood, but the epithelial layer is lost and replaced by fibroblastic scar tissue (Ward *et al*., 2005). One of the first features of this disease is apoptosis of the airway epithelial cells (Alho *et al*., 2003) and may be mediated by chronically activated $CD8^+$ T-cells in the epithelial layer (Ward *et al*., 2005).

Damage to the airway epithelium is not restricted to disease states. For example, CC16 secreted by Clara cells is increased in the children attending swimming pools suggesting exposure to ozone or chlorine may alter epithelial integrity (Lagerkvist *et al*., 2004). Elite athletes and competitive rowers also demonstrate increased numbers of airway epithelial cells in induced sputum (Bonsignore *et al*., 2003; Morici *et al*., 2004) but these cells do not exhibit high expression of adhesion molecules or expression of inflammatory transcription factors. This would suggest that increased rates of airflow across the epithelium could drive mechanical damage but this does not appear to elicit an inflammatory response.

3.11 Conclusions

The airway epithelium is the protective barrier that prevents the underlying mucosa becoming a target for damage caused by tobacco smoke, pollutants and infectious agents. In the disease state, the airway epithelium is altered to become an inflammatory cell capable of producing cytokines and chemokines as well as other mediators that can perpetuate the inflammatory response. These responses appear to be disease-specific. For example, epithelial fragility and shedding seems to be associated with asthma but is not seen in COPD or CF. Whether this is due to an inherent defect in the asthmatic epithelial cell or is due to the specific type of stimulus, for example, allergen challenge and associated protease damage, is not clear. Similarly, in COPD, the airway epithelium appears to produce an exaggerated response upon exposure to cigarette smoke, the main causative factor in the disease. At present, pharmaceutical interventions to address the chronic inflammatory responses observed in both asthma and COPD have targeted the inflammatory leukocyte influxes; however, the differential hyperresponsiveness of the airway epithelium seen in these diseases may offer a potential target(s) for the treatment of these inflammatory diseases.

References

Adam E, Hansen KK, Astudillo OF, Coulon L, Bex F, Duhant X, Jaumotte E, Hollenberg MD, and Jacquet A (2006) The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *J Biol Chem*, **281**, 6910–6923.

Alho HS, Salminen US, Maasilta PK, Paakko P, and Harjula AL (2003) Epithelial apoptosis in experimental obliterative airway disease after lung transplantation. *J Heart Lung Transplant*, **22**, 1014–1022.

Ankerst J (2005) Combination inhalers containing inhaled corticosteroids and long-acting β2 agonists: improved clinical efficacy and dosing options in patients with asthma. *J Asthma*, **42**, 715–724.

Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, and Stewart GA (2002) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol*, **169**, 4572–4578.

Barnes PJ (2000a) Chronic obstructive pulmonary disease. *N Engl J Med*, **343**, 269–280.

Barnes PJ (2000b) Inhaled corticosteroids are not beneficial in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, **161**, 342–344.

Barnes PJ, Shapiro SD, and Pauwels RA (2003) Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J*, **22**, 672–688.

Bayram H, Rusznak C, Khair OA, Sapsford RJ, and Abdelaziz MM (2002) Effect of ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures of non-asthmatic and asthmatic subjects. *Clin Exp Allergy*, **32**, 1285–1292.

Beisswenger C, Platz J, Seifart C, Vogelmeier C, and Bals R (2004) Exposure of differentiated airway epithelial cells to volatile smoke in vitro. *Respiration*, **71**, 402–409.

Blomberg A, Mudway I, Svensson M, Hagenbjork-Gustafsson A, Thomasson L, Helleday R, Dumont X, Forsberg B, Nordberg G, and Bernard A (2003) Clara cell protein as a biomarker for ozone-induced lung injury in humans. *Eur Respir J*, **22**, 883–888.

Blyth DI, Pedrick MS, Savage TJ, Hessel EM, and Fattah D (1996) Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol*, **14**, 425–438.

Boland S, Baeza-Squiban A, Fournier T, Houcine O, Gendron MC, Chevrier M, Jouvenot G, Coste A, Aubier M, and Marano F (1999) Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *Am J Physiol – Lung Cell Mol Physiol*, **276**, L604–L613.

Bonsignore MR, Morici G, Vignola AM, Riccobono L, Bonanno A, Profita M, Abate P, Scichilone N, Amato G, Bellia V, and Bonsignore G (2003) Increased airway inflammatory cells in endurance athletes: what do they mean? *Clin Exp Allergy*, **33**, 14–21.

Bosson J, Stenfors N, Bucht A, Helleday R, Pourazar J, Holgate ST, Kelly FJ, Sandstrom T, Wilson S, Frew AJ, and Blomberg A (2003) Ozone-induced bronchial epithelial cytokine expression differs between healthy and asthmatic subjects. *Clin Exp Allergy*, **33**, 777–782.

Bousquet J, Jeffery PK, Busse WW, Johnson M, and Vignola AM (2000) Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med*, **161**, 1720–1745.

Bowler RP, Barnes PJ, and Crapo JD (2004) The role of oxidative stress in chronic obstructive pulmonary disease. *COPD*, **1**, 255–277.

Chmiel JF and Davis PB (2003) State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? *Respir Res*, **4**, 8.

Churg A and Wright JL (2002) Airway wall remodeling induced by occupational mineral dusts and air pollutant particles. *Chest*, **122**, 306S–309S.

Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, Sahagan BG, and Neote K (1998) Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med*, **187**, 2009–2021.

Culpitt SV, Maziak W, Loukidis S, Nightingale JA, Matthews JL, and Barnes PJ (1999) Effect of high dose inhaled steroid on cells, cytokines, and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, **160**, 1635–1639.

Currie GP, Lee DKC, and Wilson AM (2005) Effects of dual therapy with corticosteroids plus long acting [beta]2-agonists in asthma. *Respir Med*, **99**, 683–694.

de Bentzmann S, Roger P, Dupuit F, Bajolet-Laudinat O, Fuchey C, Plotkowski MC, and Puchelle E (1996a) Asialo GM1 is a receptor for *Pseudomonas aeruginosa* adherence to regenerating respiratory epithelial cells. *Infect Immun*, **64**, 1582–1588.

de Bentzmann S, Roger P, and Puchelle E (1996b) *Pseudomonas aeruginosa* adherence to remodelling respiratory epithelium. *Eur Respir J*, **9**, 2145–2150.

Delgado MA, Poschet JF, and Deretic V (2006) Nonclassical pathway of *Pseudomonas aeruginosa* DNA-induced interleukin-8 secretion in cystic fibrosis airway epithelial cells. *Infect Immun*, **74**, 2975–2984.

Donaldson GC, Seemungal TA, Bhowmik A, and Wedzicha JA (2002) Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax*, **57**, 847–852.

Donnelly LE and Barnes PJ (2006) Chemokine receptors as therapeutic targets in chronic obstructive pulmonary disease. *Trends Pharmacol Sci*, **27**, 546–553.

Dorscheid DR, Wojcik KR, Sun S, Marroquin B, and White SR (2001) Apoptosis of airway epithelial cells induced by corticosteroids. *Am J Respir Crit Care Med*, **164**, 1939–1947.

Ekici A, Ekici M, Kurtipek E, Akin A, Arslan M, Kara T, Apaydin Z, and Demir S (2005) Obstructive airway diseases in women exposed to biomass smoke. *Environ Res*, **99**, 93–98.

Erjefalt JS, Korsgren M, Nilsson MC, Sundler F, and Persson CG (1997) Association between inflammation and epithelial damage-restitution processes in allergic airways in vivo. *Clin Exp Allergy*, **27**, 1344–1355.

Glader P, Moller S, Lilja J, Wieslander E, Lofdahl CG, and von Wachenfeldt K (2006) Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. *Respir Med*, **100**, 818–827.

GOLD (2001) Global Initiative for Chronic Obstructive Pulmonary Disease. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease NHLB/WHO Workshop Report. NIH Publication No. 2701.

Goldie RG and Pedersen KE (1995) Mechanisms of increased airway microvascular permeability: role in airway inflammation and obstruction. *Clin Exp Pharmacol Physiol*, **22**, 387–396.

Gorter AD, Eijk PP, van Wetering S, Hiemstra PS, Dankert J, and van Alphen L (1998) Stimulation of the adherence of *Haemophilus influenzae* to human lung epithelial cells by antimicrobial neutrophil defensins. *J Infect Dis*, **178**, 1067–1074.

Grunstein MM, Veler H, Shan X, Larson J, Grunstein JS, and Chuang S (2005) Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle. *J Allergy Clin Immunol*, **116**, 94–101.

Heijink IH, Kies PM, van Oosterhout AJM, Postma DS, Kauffman HF, and Vellenga E (2006) Der p, IL-4 and TGF-{beta} cooperatively induce EGFR-dependent TARC expression in airway epithelium. *Am J Respir Cell Mol Biol*, **36**, 351–359.

Herbert CA, King CM, Ring PC, Holgate ST, Stewart GA, Thompson PJ, and Robinson C (1995) Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *Am J Respir Cell Mol Biol*, **12**, 369–378.

Hodge S, Hodge G, Holmes M, and Reynolds PN (2005) Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *Eur Respir J*, **25**, 447–454.

Hong JH, Lee SI, Kim KE, Yong TS, Seo JT, Sohn MH, and Shin DM (2004) German cockroach extract activates protease-activated receptor 2 in human airway epithelial cells. *J Allergy Clin Immunol*, **113**, 315–319.

Hozumi A, Nishimura Y, Nishiuma T, Kotani Y, and Yokoyama M (2001) Induction of MMP-9 in normal human bronchial epithelial cells by TNF-alpha via NF-kappa B-mediated pathway. *Am J Physiol Lung Cell Mol Physiol*, **281**, L1444–L1452.

Innes AL, Woodruff PG, Ferrando RE, Donnelly S, Dolganov GM, Lazarus SC, and FAHY JV (2006) Epithelial mucin stores are increased in the large airways of smokers with airflow obstruction. *Chest*, **130**, 1102–1108.

Ito K, Lim S, Caramori G, Chung KF, Barnes PJ, and Adcock IM (2001) Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. *FASEB J*, **15**, 1110–1112.

Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, and Adcock IM (2006) Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J Exp Med*, **203**, 7–13.

Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, and Kay AB (1989) Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis*, **140**, 1745–1753.

Jeffery PK (2004) Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc*, **1**, 176–183.

Joos GF, Germonpre PR, Kips JC, Peleman RA, and Pauwels RA (1994) Sensory neuropeptides and the human lower airways: present state and future directions. *Eur Respir J*, **7**, 1161–1171.

Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, and Voelkel NF (2000) Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Investig*, **106**, 1311–1319.

Kauffman H, Tamm M, Timmerman JA, and Borger P (2006) House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. *Clin Mol Allergy*, **4**, 5.

Keatings VM, Collins PD, Scott DM, and Barnes PJ (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med*, **153**, 530–534.

Lagerkvist BJ, Bernard A, Blomberg A, Bergstrom E, Forsberg B, Holmstrom K, Karp K, Lundstrom NG, Segerstedt B, Svensson M, and Nordberg G (2004) Pulmonary epithelial integrity in children: relationship to ambient ozone exposure and swimming pool attendance. *Environ Health Perspect*, **112**, 1768–1771.

Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, and Lo D (1999) Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol*, **162**, 2477–2487.

Lindberg A, Eriksson B, Larsson LG, Ronmark E, Sandstrom T, and Lundback B (2006) Seven-year cumulative incidence of COPD in an age-stratified general population sample. *Chest*, **129**, 879–885.

Luppi F, Aarbiou J, van Wetering S, Rahman I, de Boer WI, Rabe KF, and Hiemstra PS (2005) Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells in vitro: role of glutathione. *Respir Res*, **6**, 140.

Marwick JA, Kirkham P, Gilmour PS, Donaldson K, MacNee W, and Rahman I (2002) Cigarette smoke-induced oxidative stress and TGF-β1 increase p21waf1/cip1 expression in alveolar epithelial cells. *Ann N Y Acad Sci*, **973**, 278–283.

Mattoli S (2001) Allergen-induced generation of mediators in the mucosa. *Environ Health Perspect*, **109** Suppl 4, 553–557.

Meagher LC, Cousin JM, Seckl JR, and Haslett C (1996) Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol*, **156**, 4422–4428.

Melis M, Siena L, Pace E, Gjomarkaj M, Profita M, Pirazzoli A, Todaro M, Stassi G, Bonsignore G, and Vignola AM (2002) Fluticasone induces apoptosis in peripheral T-lymphocytes: a comparison between asthmatic and normal subjects. *Eur Respir J*, **19**, 257–266.

Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, and Rennard SI (1997) Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med*, **155**, 1770–1776.

Mohan K, Ding Z, Hanly J, and Issekutz TB (2002) IFN-gamma-inducible T cell alpha chemoattractant is a potent stimulator of normal human blood T lymphocyte transendothelial migration: differential regulation by IFN-gamma and TNF-alpha. *J Immunol*, **168**, 6420–6428.

Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, Kilty I, and Rahman I (2004) Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-& kappa;B activation and proinflammatory cytokine release in alveolar epithelial cells. *FASEB J*, 04-1506fje.

Morici G, Bonsignore MR, Zangla D, Riccobono L, Profita M, Bonanno A, Paterno A, Di Giorgi R, Mirabella F, Chimenti L, Benigno A, Vignola AM, Bellia V, Amato G, and Bonsignore G (2004) Airway cell composition at rest and after an all-out test in competitive rowers. *Med Sci Sports Exerc*, **36**, 1723–1729.

Oddera S, Silvestri M, Balbo A, Jovovich BO, Penna R, Crimi E, and Rossi GA (1996) Airway eosinophilic inflammation, epithelial damage, and bronchial hyperresponsiveness in patients with mild–moderate, stable asthma. *Allergy*, **51**, 100–107.

Ordonez C, Ferrando R, Hyde DM, Wong HH, and Fahy JV (2000) Epithelial desquamation in asthma. Artifact or pathology? *Am J Respir Crit Care Med*, **162**, 2324–2329.

Ordonez CL, Khashayar R, Wong HH, Ferrando RE, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, and Fahy JV (2001) Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med*, **163**, 517–523.

O'Sullivan S, Cormican L, Burke CM, and Poulter LW (2004) Fluticasone induces T cell apoptosis in the bronchial wall of mild to moderate asthmatics. *Thorax*, **59**, 657–661.

Page K, Strunk VS, and Hershenson MB (2003) Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. *J Allergy Clin Immunol*, **112**, 1112–1118.

Page K, Hughes VS, Bennett GW, and Wong HR (2006) German cockroach proteases regulate matrix metalloproteinase-9 in human bronchial epithelial cells. *Allergy*, **61**, 988–995.

Patel IS, Roberts NJ, Lloyd-Owen SJ, Sapsford RJ, and Wedzicha JA (2003) Airway epithelial inflammatory responses and clinical parameters in COPD. *Eur Respir J*, **22**, 94–99.

Perez-Padilla R, Regalado J, Vedal S, Pare P, Chapela R, Sansores R, and Selman M (1996) Exposure to biomass smoke and chronic airway disease in Mexican women. A case-control study. *Am J Respir Crit Care Med*, **154**, 701–706.

Piacentini GL, Vicentini L, Mazzi P, Chilosi M, Martinati L, and Boner AL (1998) Mite-antigen avoidance can reduce bronchial epithelial shedding in allergic asthmatic children. *Clin Exp Allergy*, **28**, 561–567.

Pierrou S, Broberg P, O'Donnell R, Pawlowski K, Virtala R, Lindqvist E, Richter A, Wilson S, Angco G, Moller S, Bergstrand H, Koopmann W, Wieslander E, Stromstedt PE, Holgate S, Davies D, Lund J, and Djukanovic R (2006) Expression of genes involved in oxidative stress responses in airway epithelial cells of COPD smokers. *Am J Respir Crit Care Med*, **175**, 577–586.

Rahman I and MacNee W (1999) Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *AJP – Lung Cell Mol Physiol*, **277**, L1067–L1088.

Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, and Kay AB (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med*, **326**, 298–304.

Robinson DS, Bentley AM, Hartnell A, Kay AB, and Durham SR (1993) Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. *Thorax*, **48**, 26–32.

Rogers DF (2002) Airway goblet cell hyperplasia in asthma: hypersecretory and anti-inflammatory? *Clin Exp Allergy*, **32**, 1124–1127.

Rusznak C, Sapsford RJ, Devalia JL, Justin John R, Hewitt EL, Lamont AG, Wood AJ, Shah SS, Davies RJ, and Lozewicz S (1999) Cigarette smoke potentiates house dust mite allergen-induced increase in the permeability of human bronchial epithelial cells in vitro. *Am J Respir Cell Mol Biol*, **20**, 1238–1250.

Rusznak C, Mills PR, Devalia JL, Sapsford RJ, Davies RJ, and Lozewicz S (2000) Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol*, **23**, 530–536.

Rusznak C, Sapsford RJ, Devalia JL, Shah SS, Hewitt EL, Lamont AG, Davies RJ, and Lozewicz S (2001) Interaction of cigarette smoke and house dust mite allergens on inflammatory mediator release from primary cultures of human bronchial epithelial cells. *Clin Exp Allergy*, **31**, 226–238.

Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallesco G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM (1999) CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, **160**, 711–717.

Saetta M, Mariani M, Panina-Bordignon P, Turato G, Buonsanti C, Baraldo S, Bellettato CM, Papi A, Corbetta L, Zuin R, Sinigaglia F, and Fabbri LM (2002) Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, **165**, 1404–1409.

Sajjan U, Keshavjee S, and Forstner J (2004) Responses of well-differentiated airway epithelial cell cultures from healthy donors and patients with cystic fibrosis to *Burkholderia cenocepacia* infection. *Infect Immun*, **72**, 4188–4199.

Sajjan US, Jia Y, Newcomb DC, Bentley JK, Lukacs NW, LiPuma JJ, and Hershenson MB (2006) *H. influenzae* potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. *FASEB J*, **20**, 2121–2123.

Sanmugalingham D, De Vries E, Gauntlett R, Symon FA, Bradding P, and Wardlaw AJ (2000) Interleukin-5 enhances eosinophil adhesion to bronchial epithelial cells. *Clin Exp Allergy*, **30**, 255–263.

Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA, Hamid Q, and Luster AD (1999) The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. *J Immunol*, **162**, 3549–3558.

Schulz C, Kratzel K, Wolf K, Schroll S, Kohler M, and Pfeifer M (2004) Activation of bronchial epithelial cells in smokers without airway obstruction and patients with COPD. *Chest*, **125**, 1706–1713.

Sexton DW, Al Rabia M, Blaylock MG, and Walsh GM (2004) Phagocytosis of apoptotic eosinophils but not neutrophils by bronchial epithelial cells. *Clin Exp Allergy*, **34**, 1514–1524.

Shimura S, Andoh Y, Haraguchi M, and Shirato K (1996) Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma. *Eur Respir J*, **9**, 1395–1401.

Shinbori T, Walczak H, and Krammer PH (2004) Activated T killer cells induce apoptosis in lung epithelial cells and the release of pro-inflammatory cytokine TNF-alpha. *Eur J Immunol*, **34**, 1762–1770.

Tabary O, Corvol H, Boncoeur E, Chadelat K, Fitting C, Cavaillon JM, Clement A, and Jacquot J (2006) Adherence of airway neutrophils and inflammatory response are increased in CF airway epithelial cell–neutrophil interactions. *AJP – Lung Cell Mol Physiol*, **290**, L588–L596.

Takafuji S, Ohtoshi T, Takizawa H, Tadokoro K, and Ito K (1996) Eosinophil degranulation in the presence of bronchial epithelial cells. Effect of cytokines and role of adhesion. *J Immunol*, **156**, 3980–3985.

Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Nakahara K, and Umeda A (2001) Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med*, **163**, 1476–1483.

Tao F, Gonzalez-Flecha B, and Kobzik L (2003) Reactive oxygen species in pulmonary inflammation by ambient particulates. *Free Radical Biol Med*, **35**, 327–340.

Tateishi K, Motojima S, Kushima A, Fukuda T, and Makino S (1996) Comparison between allergen-induced and exercise-induced asthma with respect to the late asthmatic response, airway responsiveness, and Creola bodies in sputum. *Ann Allergy Asthma Immunol*, **77**, 229–237.

Tomita K, Barnes PJ, and Adcock IM (2003) The effect of oxidative stress on histone acetylation and IL-8 release. *Biochem Biophys Res Commun*, **301**, 572–577.

Trautmann A, Schmid-Grendelmeier P, Kruger K, Crameri R, Akdis M, Akkaya A, Brocker EB, Blaser K, and Akdis CA (2002) T cells and eosinophils cooperate in the induction of bronchial epithelial cell apoptosis in asthma. *J Allergy Clin Immunol*, **109**, 329–337.

Traves SL, Culpitt SV, Russell RE, Barnes PJ, and Donnelly LE (2002) Increased levels of the chemokines GROα and MCP-1 in sputum samples from patients with COPD. *Thorax*, **57**, 590–595.

Tse R, Marroquin BA, Dorscheid DR, and White SR (2003) β-Adrenergic agonists inhibit corticosteroid-induced apoptosis of airway epithelial cells. *Am J Physiol – Lung Cell Mol Physiol*, **285**, L393–L404.

Tuder RM, Zhen L, Cho CY, Taraseviciene-Stewart L, Kasahara Y, Salvemini D, Voelkel NF, and Flores SC (2003) Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. *Am J Respir Cell Mol Biol*, **29**, 88–97.

Venaille TJ, Ryan G, and Robinson BW (1998) Epithelial cell damage is induced by neutrophil-derived, not pseudomonas-derived, proteases in cystic fibrosis sputum. *Respir Med*, **92**, 233–240.

Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, and Robinson C (1999) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest*, **104**, 123–133.

Wang B, Cleary PP, Xu H, and Li JD (2003) Up-regulation of interleukin-8 by novel small cytoplasmic molecules of nontypeable *Haemophilus influenzae* via p38 and extracellular signal-regulated kinase pathways. *Infect Immun*, **71**, 5523–5530.

Wang H, Liu X, Umino T, Skold CM, Zhu Y, Kohyama T, Spurzem JR, Romberger DJ, and Rennard SI (2001) Cigarette smoke inhibits human bronchial epithelial cell repair processes. *Am J Respir Cell Mol Biol*, **25**, 772–779.

Wang SZ and Forsyth KD (2000) The interaction of neutrophils with respiratory epithelial cells in viral infection. *Respirology*, **5**, 1–9.

Wang SZ, Xu H, Wraith A, Bowden JJ, Alpers JH, and Forsyth KD (1998) Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus. *Eur Respir J*, **12**, 612–618.

Ward C, Forrest IA, Murphy DM, Johnson GE, Robertson H, Cawston TE, Fisher AJ, Dark JH, Lordan JL, Kirby JA, and Corris PA (2005) Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients. *Thorax*, **60**, 865–871.

Watanabe T, Jono H, Han J, Lim DJ, and Li JD (2004) Synergistic activation of NF-κ B by nontypeable *Haemophilus influenzae* and tumor necrosis factor α. *Proc Natl Acad Sci*, **101**, 3563–3568.

Wenzel S (2003) Mechanisms of severe asthma. *Clin Exp Allergy*, **33**, 1622–1628.

White SR and Dorscheid DR (2002) Corticosteroid-induced apoptosis of airway epithelium: a potential mechanism for chronic airway epithelial damage in asthma. *Chest*, **122**, 278S–284.

Wickenden JA, Clarke MCH, Rossi AG, Rahman I, Faux SP, Donaldson K, and MacNee W (2003) Cigarette smoke prevents apoptosis through inhibition of caspase activation and induces necrosis. *Am J Respir Cell Mol Biol*, **29**, 562–570.

Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, and Tetley TD (2004) Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. *Am J Respir Cell Mol Biol*, **30**, 500–509.

Wong CK, Ho CY, Ko FW, Chan CH, Ho AS, Hui DS, and Lam CW (2001) Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol*, **125**, 177–183.

Wong CK, Li MLY, Wang CB, Ip WK, Tian YP, and Lam CWK (2006) House dust mite allergen Der p 1 elevates the release of inflammatory cytokines and expression of adhesion molecules in co-culture of human eosinophils and bronchial epithelial cells. *Internat Immunol*, **18**, 1327–1335.

Wu R, Martin WR, Robinson CB, St George JA, Plopper CG, Kurland G, Last JA, Cross CE, McDonald RJ, and Boucher R (1990) Expression of mucin synthesis and secretion in human tracheobronchial epithelial cells grown in culture. *Am J Respir Cell Mol Biol*, **3**, 467–478.

Yokohori N, Aoshiba K, and Nagai A (2004) Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. *Chest*, **125**, 626–632.

Yoshihara S, Yamada Y, Abe T, Linden A, and Arisaka O (2006) Association of epithelial damage and signs of neutrophil mobilization in the airways during acute exacerbations of paediatric asthma. *Clin Exp Immunol*, **144**, 212–216.

4

Epithelial Repair and Function

Carl G. A. Persson[1], **Morgan Andersson**[2] and **Lena Uller**[3]
[1] *Department of Clinical and Experimental Pharmacology, Lund University, Sweden*
[2] *Lund University Hospital, Sweden*
[3] *School of Medicine, Southampton General Hospital, UK*

4.1 Brief introduction to epithelial shedding-repair and associated functions in health and disease

To some extent, shedding of epithelial cells from the airway clearly can occur without resulting in grave mucosal derangement and onset of major repair processes. Thus, single epithelial cells can be shed by mechanisms apparently involving unimpeded integrity of the epithelial lining. Even clusters of columnar epithelium can be shed without leaving any evident open gaps in the epithelial lining. In the latter circumstance, the remaining basal cells promptly develop a structural barrier above which restitution of lost columnar cells takes place. A metaplastic epithelium with multiple cell layers, as can be seen in inflammatory airway diseases, could also be expected to lose superficial epithelial cells without appreciable loss of its composite barrier. Thus, epithelial shedding is not incompatible with well maintained mucosal functions. This aspect is further underscored by the tendency of epithelial cell loss to be exceedingly patchy. By inference, therefore, epithelial cell sacrifice could, in part, be viewed as a component of first line airway defence to infectious and toxic insults.

Studies involving 'shedding-like' loss in vivo of all epithelial cells in a small area indicate that the denuded, but uninjured, epithelial basement membrane is not left naked for long. In an area where all epithelial cells are lost. bulk plasma immediately covers the exposed basement membrane. In the area of damage unfiltered plasma is promptly extravasated through newly formed endothelial gaps in venular walls of a profuse subepithelial microcirculation. The extravasated plasma moves across a pervious basement membrane forming a gel over the entire denuded area. The gel constitutes a provisional, not very tight, but still important cover. Together with locally accumulated granulocytes and other leukocytes, the plasma-derived gel provides protection as well as a suitable milieu for speedy progress of epithelial repair.

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

It is of note that a microcirculation-derived in vivo milieu is not always considered in cell culture studies, where individual molecular factors of potential importance for epithelial repair processes are usually being explored. Following denudation, epithelial cells of all phenotypes (including ciliated cells) neighbouring the denuded area promptly dedifferentiate and migrate rapidly to close the wound.

As soon as a new primitive epithelial cell barrier has been established, the plasma-derived and granulocyte-rich gel cover is resolved or shed by mixing with airway secretions/exudates. The presence of granulocytes and plasma proteins in the airway lumen also characterize asthma and chronic obstructive pulmonary disease (COPD), especially during exacerbations. Importantly, both granulocytes and bulk plasma proteins also move across a normal epithelial lining. The plasticity of the intact, pseudostratified epithelial lining cells allows unidirectional, paracellular flux of unfiltered plasma, as well as passage of granulocytes into the airway lumen. The capacity to swiftly and non-injuriously permit passage of these major components of host defence represents a significant contribution of intact airway epithelium to innate immune function in health and disease. Furthermore, recent data suggest that non-injurious egress of cells across an intact epithelial lining has a major role in eliminating granulocytes from inflamed airway tissues. New concepts regarding non-injurious elimination of inflammatory cells from airway tissues across an intact epithelial lining including its relation to occurrence and roles of apoptosis of these cells are reviewed elsewhere (Uller *et al*., 2006).

It has been demonstrated that epithelial shedding-restitution processes alone can evoke several of the pathophysiological and remodelling features of inflammatory airway diseases. These in vivo findings underpin the possibility of a central role of epithelial injury and repair in the pathogenesis of asthma (Persson *et al*., 1996), and suggest that events associated with simple epithelial repair, in part, can be compared to activation of the epithelial mesenchymal trophic unit that is operates during lung development (Demayo *et al*., 2002).

Interestingly, a commonly expected result of epithelial shedding, ‘increased permeability’, may not be prominent in vivo. Patchiness of the injury, together with quick repair, may explain why an epithelial shedding disease such as asthma is not functionally characterized by increased permeability to inhaled molecules (Persson *et al*., 1995). The fact that plasma proteins appear on the mucosal surface is, however, a different matter and cannot be interpreted as reflecting increased permeability of the epithelial lining. Epithelial mechanisms involved in swift, unidirectional luminal entry of extravasated, unfiltered plasma proteins across the intact airway mucosa are also reviewed elsewhere (Persson *et al*., 2002).

The above introductory comments are largely based on observations in vivo. By contrast, work on epithelial mechanisms is now dominated by in vitro approaches providing new information on the molecular biology and pharmacology of repair of injured cell cultures. In the early days of culture studies, readers were frequently reminded of shortcomings (as well as advantages) of the in vitro possibilities. It must be reiterated that several central features, including cell phenotypes, molecular milieus, and dynamics of local and external influences cannot be fully mimicked in cell cultures. Despite this, however, these approaches offer important opportunities for exploring reductive mechanisms. Without ignoring the problematic issue of whether in vitro observations translate to the in vivo situation (Persson *et al*., 2001), selected in vitro data will be considered in this chapter on repair and function of the pseudostratified airway epithelium in vivo.

4.2 Repair following shedding of single columnar epithelial cells and following shedding of clusters of columnar cells

The occurrence of epithelial cells in asthmatic sputa was described as long ago as the latter half of the 1800s. Strikingly, ciliated epithelial cells occurred both alone and as clusters of epithelial cells, neither of which were associated with basal cells (Persson, 1997). Using electron microscopy to examine bronchoalveolar lavage fluid, Montefort *et al*. (1992) calculated that the number of free epithelial cells was more numerous than clusters of epithelial cells in samples obtained either from healthy or asthmatic individuals. Additionally, they demonstrated that it was extremely rare to find basal cells attached to the shed columnar cells. In allergen-challenged airway of allergic guinea-pigs, patchy areas of epithelial injury-repair characteristically exhibited loss of columnar cells, but the bottom of each such epithelial crater retained a complete layer of 'basal' cells (Erjefält *et al*., 1997a). Hence, it appears that the junctional desmosomal attachment between columnar and basal cells is what is regularly lost at shedding.

4.2.1 Loss of single epithelial cells

The loss of single columnar epithelial cells (Figure 4.1(a)) is likely a frequent event in health and disease, so the consequences of single cell loss is of interest. However, this phenomenon is poorly documented. This probably reflects both the quickness with which the loss may be completed in each instance and the speed by which the space that was occupied by the lost cell is fully closed. Occasional observations by scanning electron microscopy of single columnar cells leaving the airway surface suggest the possibility that the gap already begins to close during the shedding process. Such a mechanism would explain why small gaps, reflecting loss of single cells, have not been widely reported. For example, such

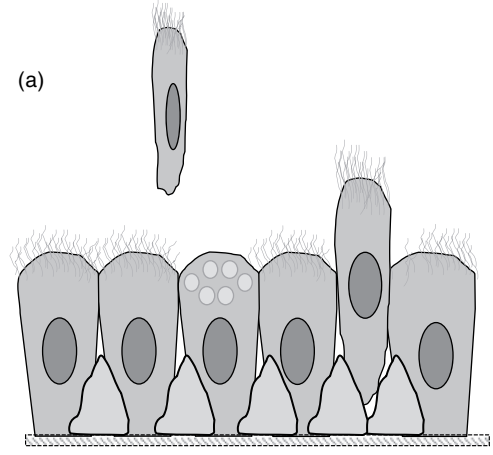


Figure 4.1 (a) Loss of single epithelial cells: 'Tight as they go'. (b) Patchy loss of clusters of columnar epithelial cells: after losing their cover of columnar cells, basal cells promptly create a new barrier. (c) Patchy areas of complete shedding/denudation: 'hot spots of speedy epithelial repair along with defence, inflammatory, and remodelling reactions'

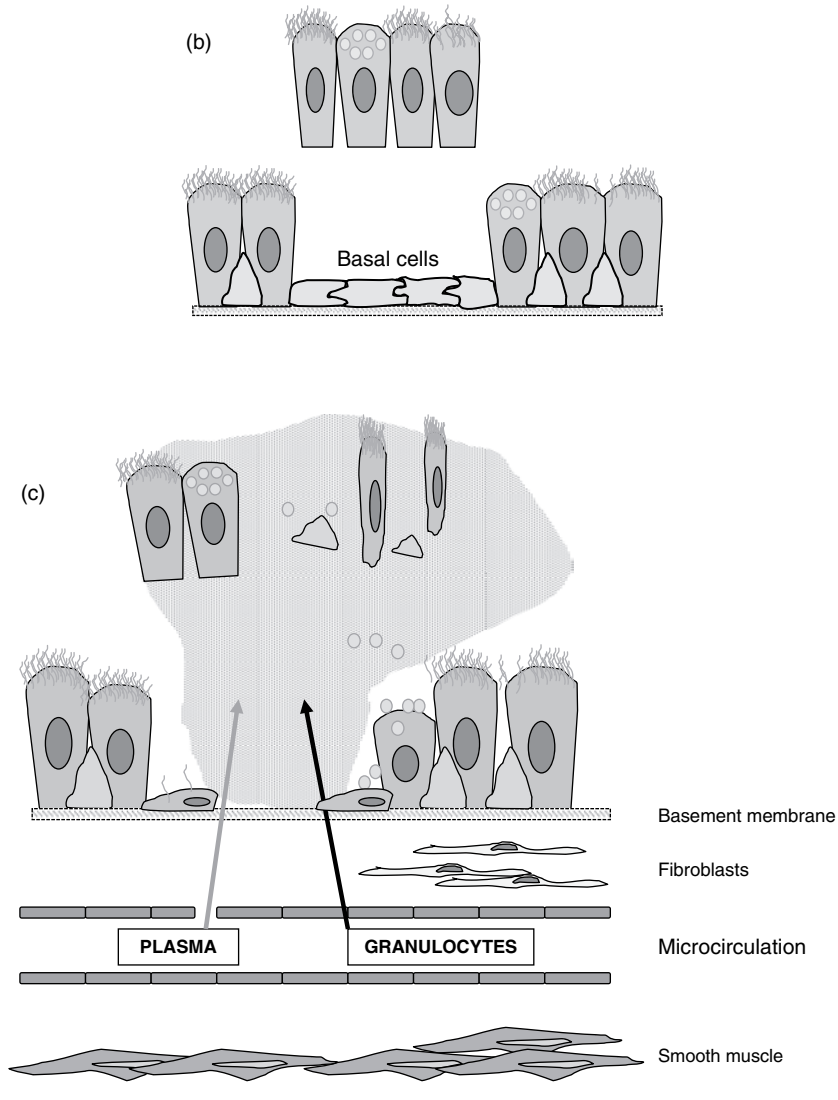


Figure 4.1 (Continued)

single-cell gaps were not detected in carefully scanned whole-mounts of an airway surface where allergen challenge had evoked significant shedding-repair processes in vivo (Erjefält *et al*., 1997a). The single-cell loss perhaps may be likened to epithelial passage of individual leukocytes in vivo. Thousands of eosinophils have been demonstrated to traverse the intact, pseudostratified epithelial lining in vivo per min and per square centimetre of airway surface without leaving any detectable apical traces of their paracellular routes into the airway lumen (Erjefält *et al*., 2004). The epithelium in vivo also regains integrity immediately after letting through an acute efflux of unfiltered plasma, including its largest proteins (Luts *et al*., 1990).

Repair of single-cell epithelial defects has been examined in epithelial monolayers (Florian *et al*., 2002). A potential limitation of such studies is that artificial destruction of a single cell in epithelial cultures, and the ensuing closure of the lesion-induced gap, may not necessarily mimic the processes involved in the 'spontaneous' shedding of an endogenously detached single epithelial cell in vivo. In addition, in vitro findings may generally underestimate the speed of epithelial restitution. For example, after scraping or stab-induced injury to cell cultures the onset of repair is delayed and the speed of cell migration to cover the wounds is initially slow (Zahm *et al*., 1991). By contrast, provided the basement membrane is unharmed, in vivo repair after denudation starts immediately and the speed of repair (rate of spreading and migration of neighbouring cells) is highest (3 μm/min) during the initial and most critical minutes after epithelial cell loss (Erjefält *et al*., 1995a). Hence, it is possible that restitution of single-cell defects in epithelial layers also differ in speed in vitro compared to in vivo. Yet in vitro observations demonstrate a reasonably quick formation of tight junctions, as demonstrated both functionally and through appearance and turnover of junction proteins (ZO-1 and occludin) in such epithelial lesions (Florian *et al*., 2002). The in vitro work further suggests a role for actin (the polymerization of which is antagonized by cytochalasin D) in migration of 'repair cells' and in a 'purse string' mode of closure of single epithelial cell lesions (Florian *et al*., 2002, Zahm *et al*., 1991). Further study is needed to determine to what extent single epithelial cells are lost in vivo in response to various insults, and whether there is a requirement for repair in these events beyond a maintained seal around the individual columnar cell that is being shed into the airway lumen.

4.2.2 Apoptosis in vitro

A purse string mechanism may also be initiated when single epithelial cells in culture become apoptotic (Florian *et al*., 2002). Apoptosis and its sequelae, again, appear to be a field where in vitro observations frequently fail to translate into in vivo systems (Uller *et al*., 2006). So far, there is little evidence for involvement of columnar or basal epithelial cell apoptosis (as properly defined by morphology) prior to shedding events in vivo. By contrast, apoptosis of alveolar lining cells has been demonstrated and has been considered to be implicated in development of emphysema (Imai *et al*., 2005). It is of note that observations derived using lung parenchyma can not *a priori* be extended to the all-important small airway changes in COPD and asthma. Similarly, nothing can be deduced about occurrence of apoptosis in the airway mucosa from observations that cells in the airway lumen, whether they are shed epithelial cells or egressed granulocytes, readily undergo apoptosis to varying extents before being finally eliminated via mucociliary clearance (Uller *et al*., 2006). The resistance of the airway epithelial lining in vivo to pro-apoptotic stimuli, such as ligands of FAS receptors (Uller *et al*., 2005), underscores our need to gain knowledge regarding the actual occurrence of epithelial apoptosis in health and disease. Such information is pivotal for appraisal of medical hypotheses on the roles of epithelial death and repair in asthma, which currently are largely based on molecular biology data.

4.2.3 Loss of clusters of columnar cells

The second most common type of shedding appears to be loss of sheets of joined columnar epithelial cells (Figure 4.1(b)). As repeatedly recorded in asthmatic sputa since the 1800s, columnar cells can stick together even after being shed and mixed with airway secretions and

exudates (Persson, 1997). It also has been repeatedly observed that asthmatic airway, in part, are covered by squamous epithelial cells without any attached columnar cells. Accordingly, these remaining cells have been called basal cells. However, they could equally well be flattened 'repair' epithelial cells moving in to cover denuded areas. Cells covering the bottom of epithelial craters, where columnar cells are lacking due to allergen challenge (Erjefält *et al*., 1997a), could thus be transformed basal cells or they could be rapidly dedifferentiated columnar epithelial cells that have quickly migrated from areas adjacent to the site of damage. At the time of these studies, there was little specific information as to what changes occurred to basal cells upon loss of their cover of columnar epithelium. A method was, therefore, developed to explore, in some detail, effects of removal of columnar cells on remaining basal cells. Tracheobronchial and nasal airway tissues with an intact mucosa and sub-mucosa were used immediately after being surgically removed from animals and humans. The mucosal surface was allowed to dry for one minute – just enough to make a drop of tissue adhesive glue stick to epithelial apices. Another half-minute was allowed for hardening of the glue which then was very gently removed, together with attached cells, by a rolling movement. Thus, it was possible to selectively remove sheets of columnar epithelium. The remaining tissue was incubated under conditions selected to approximate the in vivo milieu. This technique was successful in that it did not lead to loss of basal cells, and the immediate appearance of the remaining basal cells was similar to their drop- or cobblestone-appearance seen in an intact epithelium. Interestingly, within 20 min the basal cells underwent a dramatic transformation. They had spread and become exceedingly flat. The effect was that they fully covered the space that initially existed between the original cobble-like basal cells. Interdigitating cytoplasmatic protrusions also occurred and characterized the newly created basal cell borders. After losing their cover of columnar epithelium the basal cells thus promptly produced a novel barrier structure. This ability of basal cells did not differ between human and guinea-pig airway, nor did it differ between human nasal and bronchial airway (Erjefält *et al*., 1997b). Admittedly, the experiment was carried out ex vivo and the removal of columnar cells, however gentle, may well have stretched and stressed the basal cells in a manner unlike the natural shedding of a sheet of columnar cells under in vivo conditions. The speed of the change ex vivo was, nevertheless, remarkable. In analogy with other data (see above), it may be speculated that even higher speeds of basal cell barrier formation than observed in the ex vivo study may take place in vivo. It is currently not known to what extent shedding of only columnar cells can cause airway pathophysiological and remodelling effects similar to those evoked by epithelial denudation–restitution events.

4.3 Epithelial denudation

A century ago, the issue of whether epithelial denudation was a unifying characteristic of asthma was already a topic of debate. Due to the possibility of artefacts induced post mortem, or at tissue handling, the balance of evidence then probably rested with pathologists who demonstrated intact epithelial linings in patients with the most severe forms of asthma (Persson, 1997). However, the debate has continued and intensified in recent years. There is now increased understanding of the problem of sampling artefacts when describing epithelial loss in biopsies obtained from asthmatic individuals (Jeffery, 1996). It also has been demonstrated that cryo-sectioning clearly produces denuded areas in allergen-challenged allergic airway where denudation evidently was not present prior to the sectioning. Interestingly, the

ease by which sectioning procedures produce denudation is greatly increased in samples with ongoing inflammation (Erjefält *et al*., 1997a), suggesting that epithelial fragility is an important characteristic. The frequent demonstration of denudation in asthma could, therefore, be interpreted as reflecting a fragile epithelial lining. Increased epithelial fragility would also be expected to increase the likelihood of the occurrence of patches of truly denuded areas in vivo. This is important because even small areas of epithelial denudation–repair can cause significant pathophysiological effects, as well as remodelling of the airway (Figure 4.1(c)).

Data emerging from in vivo studies in guinea-pigs, involving 'shedding-like' (no damage to the basement membrane and no surgery or bleeding) removal of a tiny stretch of pseudostratified epithelium of the trachea have produced information on the repair milieu, on the onset and speed of repair, on which cells participate in repair, and on pathophysiological and remodelling sequelae to epithelial denudation–restitution in vivo (Persson and Erjefält, 1997). We are now exploring the possibility of producing well controlled, and non-bleeding, epithelial removal including denudation in vivo in human polyp tissues, which have epithelial features similar to those observed in asthma and, possibly, COPD as well (unpublished observations by M. Andersson, C. Persson, and L. Uller). Until the advent of validated data from studies in human airway, this discussion will focus on the animal data.

4.3.1 Plasma exudation and granulocytes

In airway with an intact mucosa, extravasated bulk plasma can enter the lumen with only a few minutes' delay. This likely reflects the need to build up a small increase in epithelial basolateral hydrostatic pressure (about 2 cm H_2O suffices) to open one-way, valve-like, paracellular epithelial pathways into the airway lumen (Gustafsson and Persson, 1991). However, in denuded spots, extravasated plasma promptly appears on the surface of the intact basement membrane. Holes in the basement membrane (Erjefält *et al*., 1994; Howat *et al*., 2001) may be the structural correlates to this unhindered passage of plasma proteins. The microvascular permeability response to denudation is both prompt and sustained. A substantial plasma-derived gel structure is first produced to completely cover the denuded area and then the exudation continuously supplies this gel with fresh proteins. During repair of denuded areas, increased amount of a significant number of proteins, including fibronectin, fibrinogen, different growth factors etc., derive from the microcirculation (Erjefält *et al*., 1994), obviating the need for epithelial cells themselves to produce many of these proteins. The plasma proteins and their active degradation products also have chemoattractant properties explaining, in part, the ensuing accumulation of leukocytes, including many neutrophils, in the gel cover. Neutrophils in the repair gel contribute to host defence in the vulnerable areas with defective epithelium. Neutrophils may also promote repair by clearing the mucosa of necrotic epithelial cells (such as occur acutely upon exposure to ozone) which otherwise will impede the repair process (Hyde *et al*., 1999).

4.3.2 Ciliated and secretory cells dedifferentiate and become speedily migrating repair cells

Whilst plasma exudation is in continued progress, a new cell lining is quickly established. The loss of neighbouring epithelial cells, obviously provides a strong signal for repair. Thus, at the border between the denuded surface and the maintained pseudostratified epithelium, the activity is dramatic. Here the ciliated cells internalize their cilia, and the secretory cells

discharge their granules. At the same time both cell types change from a columnar to a flattened shape and start to migrate over the basement membrane. The dedifferentiated migrating cells remain dynamically attached to each other by patchy cell–cell connections. During the first 15 minutes after denudation, migration occurs at speeds of about 3 μm/min, and, in this time frame, the newly formed migratory cell sheet covers up to 60 μm wide zones of the previously denuded basement membrane. The ultrastructural evidence demonstrating that the ciliated cell is not terminally differentiated, as was previously believed, but is ready to dedifferentiate to participate in repair is corroborated by a maintained ratio between secretory and ciliated cells along the borders of the repair zone (Erjefält *et al*., 1995a). Incidentally, this role of the ciliated cell has recently been noted again by Park *et al*. (2006). As demonstrated in the mid-1990s, the efficiency of the initial, and most critical, phase of epithelial repair is clearly dependent on the ability of both ciliated and secretory epithelial cells to dedifferentiate into primitive, migrating 'repair' cells.

Epithelial damage occurring in response to inflammatory insults, such as allergen challenge, cause exceedingly patchy, almost circular, epithelial damage sites. This is the case even if the challenge is applied very uniformly over a large surface area (Erjefält *et al*., 1997a). The effects of cells dedifferentiating and migrating from all around such a site very rapidly produce a new lining of cells, making the presence of actual denudation very short-lived events in vivo. Whether extrapulmonary progenitor epithelial cells contribute to repair after patchy shedding of cells from the pseudostratified airway epithelium remains speculative.

4.3.3 Features of the initial cover of repair cells and its development into a normal fully differentiated airway epithelium

When the denuded area has received its initial primitive cell cover, the plasma-derived gel together with its additional components including granulocytes is resolved and shed. Then there is a more slow development of a normal pseudostratified epithelium. The new epithelium eventually consists of a few layers of squamous, poorly differentiated cells, as is also observed in inflammatory airway diseases. On the surface aspect, these cells display ridge-like seals. Tight junctions and desmosomes also develop. Within a few days, ciliated and secretory columnar epithelial cells appear and then, after a few more days, the phenotypic appearance of the previously denuded area is no longer distinguishable from areas that have not been denuded. The time periods involved would be dependent upon the size of the denudation. Here, the approximate times given represent the repair following denudation of an 800 μm wide epithelial path (Erjefält *et al*., 1995a). After a single denudation cycle of this artificial size, abnormally increased numbers of secretory cells was never observed.

4.3.4 Epithelial proliferation and repair

The migrating epithelial cells involved in repair do not display increased mitotic activity above that of a normal intact epithelium. However, once the initial cover is complete, these cells increase their mitotic activity 10–20-fold. Simultaneously, a more modest, fourfold increase in mitotic activity occurs in the 'old' epithelium surrounding the damaged area (Erjefält *et al*., 1995a). Cell proliferation is clearly an important component of epithelial repair, but it is not involved in the early critical phase of restitution of an epithelial cell cover. The rather sustained increase in epithelial mitotic activity makes this a potentially useful

index of repair activity to be detected in airway biopsies. Increased epithelial cell proliferation has also been reported in asthmatic and rhinitic airway. Interestingly, Kicic *et al*. (2006), working with samples of epithelial cells obtained by brushing, have demonstrated that the proliferative capacity of these cells is greater in children with mild asthma than in healthy controls. Holgate *et al*. (2003), also working with cultures of human airway epithelial cells, observed no difference between adult asthmatics and normal individuals regarding proliferation rates. Yet, based on intriguing molecular biology data and on epithelial repair data in vitro they have advanced the idea that the duration of epithelial repair is prolonged in asthma due to an imbalance between proliferation and cell survival signals. The possibility of altered features of the asthmatic epithelium impairing its own repair makes it all the more important to study actual epithelial restitution events in diseased airway in vivo. Exposure to tobacco smoke has been reported to slow down epithelial repair in animals exposed to toxic levels of naphthalene (Van Winkle *et al*., 2004), suggesting that epithelial repair defects may be involved also in the pathogenesis of COPD.

4.4 Pharmacology of epithelial repair

4.4.1 Repair-promoting factors

In recent years numerous reports have identified individual molecules of putative significance in epithelial repair. The shape changes in cells that dedifferentiate and migrate in the immediate response to epithelial removal necessarily involve activity of cytoskeleton proteins (Zahm *et al*., 1991). Thus, cell migration may be facilitated by several matrix metalloproteinases (MMPs), including MMPs 3,7, and 9, potentially via effects on cell–cell contacts and on the extracellular matrix (Buisson *et al*., 1996; Parks *et al*., 2001). Clearly, growth factors with a particular focus on the ability of EGF to stimulate proliferation, have attracted interest (Holgate *et al*., 2003). There have also been attempts to identify individual serum factors of importance for epithelial repair (Patchell and Dorscheid, 2006). Lackie and Adam (2006) have summarized in vitro findings focusing on the potential for cellular carbohydrates to enhance epithelial repair. Other agents reported to stimulate closure of epithelial wounds in vitro include adenosine agonists (Allen-Gipson *et al*., 2006), bombesin (Tan *et al*., 2006), and neutrophil defensins (Aarbiou *et al*., 2004). Hence, a rapidly growing number of autacoids remain to have their potential roles defined in in vivo studies of epithelial repair in health and disease.

4.4.2 Repair-retarding factors

Rhinovirus infections target the airway epithelium causing generation and release of major regulatory proteins (Proud and Chow, 2006). However, little has been documented regarding the reputed viral infection-induced epithelial damage. Bossios *et al*. (2005) now claim to have an in vitro epithelial system where they find cytotoxic actions of rhinovirus infection as well as delayed wound healing. TGF-beta may exemplify endogenous agents reported to attenuate (Neurohr *et al*., 2006), as well as increase (Lechapt-Zalcman *et al*., 2006), repair of wounds in epithelial cell cultures. Details regarding possible links between inflammatory mediators, developmental pathways and epithelial repair are reviewed by Demayo *et al*. (2002) in their discussion on pathogenesis of bronchopulmonary dysplasia. Of apparent concern, are the reports on negative repair effects of drugs employed chronically as local airway treatments

of obstructive airway diseases. Thus, both beta agonists and corticosteroids can impede the repair of scraping-induced 'denudation' paths in cultured layers of epithelial cells (Dorscheid *et al*., 2006; Schnackenberg *et al*., 2006). These in vitro test systems may exhibit different pharmacological features depending on the number of 'denudation' scrapings employed, and it has already been suggested that neither type of anti-asthma drug affects repair negatively in more 'chronic' cell culture experiments (Wadsworth *et al*., 2006). An urgent need emerges for in vivo evaluation of effects of airway drugs before speculation on treatment interference with epithelial repair grow out of proportion. Indeed, a potent airway steroid, given prior to denudation as well as during the repair phase, was without effect on the prompt and high-speed repair mechanisms evoked in vivo at shedding-like denudation experiments (Erjefält *et al*., 1995b).

4.5 Epithelial shedding-restitution as a causative process in airway inflammation and remodelling

4.5.1 Pathophysiology

Almost by definition, epithelial shedding beyond the loss of single columnar cells should cause some increase in mucosal permeability. However, as discussed above, the ability of the epithelium to create new barriers is so highly developed that significant shedding can occur without causing any troublesome permeability to inhaled noxious stimuli. The somewhat confusing medical history involving reports on increased, unchanged, or decreased airway permeability in airway diseases such as asthma and allergic rhinitis has been reviewed elsewhere (Persson *et al*., 1995). Suffice it to state here that ongoing inflammatory airway disease may well exhibit a decreased (sic!) inward permeability to molecules deposited on the airway surface. A decreased functional permeability may, in part, reflect entrapment of inhaled material in secretions/exudates. It is also possible to explain reduced permeability by the presence of areas of repairing epithelium because these are characterized by a reduced length of intercellular stretches (available for paracellular absorption) compared to the same area of normal epithelium.

The epithelium, its sensory innervation, and a profuse subepithelial microcirculation are common features arranged for cross-talk both in guinea-pig trachea and human airway. As may be expected, denudation immediately affected the physiology of these three juxtapositioned mucosal end organs. Thus, in the vicinity of the tracheal denudation zone (Erjefält *et al*., 1995a), and spreading further all around the large airway, the untouched epithelium displayed a dramatic acute reduction in its stored secretions. The expelled secretions act to protect the still unharmed epithelium and could also contribute to the plasma-derived gel. The secretory capacity gradually returned to normal levels in a few days. It is possible that this secretory response, in part, was mediated via the innervation. It was also noted that as early as during the proliferation phase, which followed after covering of the denuded area with migrating repairing epithelium, peptidergic nerve fibres reappeared, indicating that restitution of a normal, fully differentiated epithelium is preceded by a scattered sensory innervation. The mechanisms involved in these events, which also occur while subepithelial fibroblasts and smooth muscle are proliferating (Erjefält *et al*., 1995a), may be of a similar nature to epithelial–mesenchymal interactions that occur in developing embryonic lungs (Demayo *et al*., 2002).

As discussed above, plasma exudation emerged as an important physiological response to denudation, and was a response that was maintained throughout the phase during which a new primitive epithelial lining was established. Re-epithelialization thus occurred in a milieu enriched with plasma-derived adhesive proteins, such as fibrinogen and fibronectin, and with growth factors, including epidermal growth factor (EGF), known to be present in plasma (Persson and Erjefält, 1997). Interestingly, the expression of EGF receptors is increased in asthmatic epithelium, which has been interpreted as a sign of widespread damage and repair (Holgate *et al*., 2003). Microvascular-epithelial exudation of plasma is a hallmark of asthma and of exacerbations of COPD and has multiple properties of pathogenetic potential in these diseases (Persson *et al*., 2002).

4.5.2 Granulocytes

The morphological correlate to the extravasation of plasma is the formation of small interendothelial gaps in venules residing just beneath the denuded zone. Although extravasation of granulocytes also occurs across the venular walls, these leukocytes do not use the gaps but migrate with a maintained sealing near these gaps. Early examinations by Felix Marchand suggested that epithelial damage in severe asthma is patchy and associated with conglomerates of neutrophils, eosinophils, and fibrin (Persson and Erjefält, 1997). Such foci of epithelial injury and associated cells and proteins have also been demonstrated in guinea-pig airway subjected to allergic inflammation (Erjefält *et al*., 1997a). Thus, patchy sites of epithelial injury-repair are associated with activated neutrophils, which even emerge into the lumen as domes of clustering cells. Potentially, the neutrophilic feature of severe asthma may, in part, reflect the occurrence of epithelial injury-repair events. Eosinophils also may abound at the epithelial repair sites (Erjefält *et al*., 1996). Eosinophils have not only been implicated in damage and epithelial shedding, but these cells may also promote repair in vivo, since their granules contain growth-promoting proteins. A particular mode of eosinophil degranulation in vivo – primary cytolysis followed by tissue deposition of clusters of free extracellular granules (Persson *et al*., 2000) – is seen both in asthmatic airway and in areas of speedy epithelial repair processes in guinea-pig airway. This latter observation contributes to complicating the discussion of the eosinophil as a culprit cell in bronchial asthma.

4.5.3 Epithelial mesenchymal cross-talk and remodelling sequelae to epithelial repair

In the absence of compelling evidence of extensive epithelial disruption, Holgate *et al*. (2003) have considered that increased epithelial expression of EGF receptors reflects widespread epithelial damage-repair in asthma. These authors have further argued that a correlation between over-expression of these receptors and the thickness of the lamina reticularis links epithelial injury to an underlying remodelling response. However, there appear to be even stronger links between epithelial shedding-repair and airway remodelling. Experimental in vivo findings have directly demonstrated multiple remodelling effects associated with the epithelial restitution processes that follow upon ‘disease-like shedding’. The remodelling seen at these shedding–restitution events in vivo involves the epithelium itself, its basement membrane, the subepithelial fibroblasts, and the smooth muscle (Persson *et al*., 1996). The repairing epithelium goes through a phase of epithelial metaplasia before restitution of

a normal epithelium. However, simple shedding-like denudation, even if repeated several times, does not produce the goblet cell hyperplasia that occurs in inflamed or diseased airway. Other mechanisms than simple shedding–restitution would thus be involved in generation of an abnormal, secretory epithelial lining. Yet, repeated denudation without any additional airway inflammatory processes is sufficient for production of a significant, asthma-like thickening of the reticular basement membrane. Furthermore, even a single cycle of denudation–repair evokes markedly increased mitosis in subepithelial fibroblasts and smooth muscle cells indicating important cross-talk between epithelium and mesenchymal cells even in minor repair events. Intriguingly, disease-like remodelling mechanisms thus emanate from the mere restitution events occurring after shedding of epithelial cells from an otherwise normal and uninflamed airway mucosa. Such observations contribute to putting focus on epithelial mechanisms as a causative component in the early pathogenesis of airway diseases (Persson *et al*., 1996; Holgate *et al*., 2003; Hackett and Knight, 2007).

References

Aarbiou J, Verhoosel RM, Van Wetering S, De Boer WI, Van Krieken JH, Litvinov SV, Rabe KF and Hiemstra PS (2004) Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. *Am J Respir Cell Mol Biol* **30**: 193–201.

Allen-Gipson DS, Wong J, Spurzem JR, Sisson JH and Wyatt TA (2006) Adenosine A2A receptors promote adenosine-stimulated wound healing in bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **290**: L849–855.

Bossios A, Psarras S, Gourgiotis D, Skevaki CL, Constantopoulos AG, Saxoni-Papageorgiou P and Papadopoulos NG (2005) Rhinovirus infection induces cytotoxicity and delays wound healing in bronchial epithelial cells. *Respir Res* **6**: 114.

Buisson AC, Zahm JM, Polette M, Pierrot D, Bellon G, Puchelle E, Birembaut P and Tournier JM (1996) Gelatinase B is involved in the in vitro wound repair of human respiratory epithelium. *J Cell Physiol* **166**: 413–426.

Demayo F, Minoo P, Plopper CG, Schuger L, Shannon J and Torday JS (2002) Mesenchymal–epithelial interactions in lung development and repair: are modeling and remodeling the same process? *Am J Physiol Lung Cell Mol Physiol* **283**: L510–517.

Dorscheid DR, Patchell BJ, Estrada O, Marroquin B, Tse R and White SR (2006) Effects of corticosteroid-induced apoptosis on airway epithelial wound closure in vitro. *Am J Physiol Lung Cell Mol Physiol* **291**: L794–801.

Erjefält JS, Erjefält I, Sundler F and Persson CG (1994) Microcirculation-derived factors in airway epithelial repair in vivo. *Microvasc Res* **48**: 161–178.

Erjefält JS, Erjefält I, Sundler F and Persson CG (1995a) In vivo restitution of airway epithelium. *Cell Tissue Res* **281**: 305–316.

Erjefält JS, Erjefält I, Sundler F and Persson CG (1995b) Effects of topical budesonide on epithelial restitution in vivo in guinea pig trachea. *Thorax* **50**: 785–792.

Erjefält JS, Sundler F, Persson CG (1996) Eosinophils, neutrophils, and venular gaps in the airway mucosa at epithelial removal–restitution. *Am J Respir Crit Care Med* **153**: 1666–1674.

Erjefält JS, Korsgren M, Nilsson MC, Sundler F and Persson CG (1997a) Association between inflammation and epithelial damage-restitution processes in allergic airways in vivo. *Clin Exp Allergy* **27**: 1344–1355.

Erjefält JS, Sundler F and Persson CG (1997b) Epithelial barrier formation by airway basal cells. *Thorax* **52**: 213–217.

Erjefält JS, Uller L, Malm-Erjefält M and Persson CG (2004) Rapid and efficient clearance of airway tissue granulocytes through transepithelial migration. *Thorax* **59**: 136–143.

Florian P, Schoneberg T, Schulzke JD, Fromm M and Gitter AH (2002) Single-cell epithelial defects close rapidly by an actinomyosin purse string mechanism with functional tight junctions. *J Physiol* **545**: 485–499.

Gustafsson BG and Persson CG (1991) Asymmetrical effects of increases in hydrostatic pressure on macromolecular movement across the airway mucosa. A study in guinea-pig tracheal tube preparations. *Clin Exp Allergy* **21**: 121–126.

Hackett TL and Knight DA (2007) The role of epithelial injury and repair in the origins of asthma. *Curr Opin Allergy Clin Immunol* **7**: 63–68.

Holgate ST, Davies DE, Puddicombe S, Richter A, Lackie P, Lordan J and Howarth P (2003) Mechanisms of airway epithelial damage: epithelial–mesenchymal interactions in the pathogenesis of asthma. *Eur Respir J Suppl* **44**: 24s–29s.

Howat WJ, Holmes JA, Holgate ST and Lackie PM (2001) Basement membrane pores in human bronchial epithelium: a conduit for infiltrating cells? *Am J Pathol* **158**: 673–680.

Hyde DM, Miller LA, McDonald RJ, Stovall MY, Wong V, Pinkerton KE, Wegner CD, Rothlein R and Plopper CG (1999) Neutrophils enhance clearance of necrotic epithelial cells in ozone-induced lung injury in rhesus monkeys. *Am J Physiol* **277**: L1190–1198.

Imai K, Mercer BA, Schulman LL, Sonett JR and D'Armiento JM (2005) Correlation of lung surface area to apoptosis and proliferation in human emphysema. *Eur Respir J* **25**: 250–258.

Jeffery PK (1996) Bronchial biopsies and airway inflammation. *Eur Respir J* **9**: 1583–1587.

Kicic A, Sutanto EN, Stevens PT, Knight DA and Stick SM (2006) Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. *Am J Respir Crit Care Med* **174**: 1110–1118.

Lackie PM and Adam EC (2006) Sweet talking-cellular carbohydrates and epithelial repair. *Clin Exp Allergy* **36**: 560–562.

Lechapt-Zalcman E, Pruliere-Escabasse V, Advenier D, Galiacy S, Charriere-Bertrand C, Coste A, Harf A, d'Ortho MP and Escudier E (2006) Transforming growth factor-beta1 increases airway wound repair via MMP-2 upregulation: a new pathway for epithelial wound repair? *Am J Physiol Lung Cell Mol Physiol* **290**: L1277–1282.

Luts A, Sundler F, Erjefält I and Persson CG (1990) The airway epithelial lining in guinea pigs is intact promptly after the mucosal crossing of a large amount of plasma exudate. *Int Arch Allergy Appl Immunol* **91**: 385–388.

Montefort S, Roberts JA, Beasley R, Holgate ST and Roche WR (1992) The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects. *Thorax* **47**: 499–503.

Neurohr C, Nishimura SL and Sheppard D (2006) Activation of transforming growth factor-beta by the integrin alphavbeta8 delays epithelial wound closure. *Am J Respir Cell Mol Biol* **35**: 252–259.

Park KS, Wells JM, Zorn AM, Wert SE, Laubach VE, Fernandez LG and Whitsett JA (2006) Transdifferentiation of ciliated cells during repair of the respiratory epithelium. *Am J Respir Cell Mol Biol* **34**: 151–157.

Parks WC, Lopez-Boado YS and Wilson CL (2001) Matrilysin in epithelial repair and defense. *Chest* **120**: 36S–41S.

Patchell BJ and Dorscheid DR (2006) Repair of the injury to respiratory epithelial cells characteristic of asthma is stimulated by *Allomyrina dichotoma* agglutinin specific serum glycoproteins. *Clin Exp Allergy* **36**: 585–593.

Persson CG (1997) Centennial notions of asthma as an eosinophilic, desquamative, exudative, and steroid-sensitive disease. *Lancet* **350**: 1021–1024.

Persson CG and Erjefält JS (1997) Airway epithelial restitution after shedding and denudation. In: *The Lung*, Barnes P, Crystal RG and West JB (eds), Lippincott Raven Publishers, Philadelphia, pp. 2611–2627.

Persson CG, Andersson M, Greiff L, Svensson C, Erjefält JS, Sundler F, Wollmer P, Alkner U, Erjefält I, Gustafsson B, Linden M and Nilsson M (1995) Airway permeability. *Clin Exp Allergy* **25**: 807–814.

Persson CG, Erjefält JS, Erjefält I, Korsgren MC, Nilsson MC and Sundler F (1996) Epithelial shedding – restitution as a causative process in airway inflammation. *Clin Exp Allergy* **26**: 746–755.

Persson CG, Erjefält JS, Korsgren M, Andersson M, Greiff L, Svensson C (2000) In vivo processes of exudative airway inflammation with ultimate eosinophil activation and epithelial injury-repair. In: *Asthma and Rhinitis*, Busse WW and Holgate ST (eds), Blackwell, Boston, pp. 732–744.

Persson CG, Erjefält JS, Uller L, Andersson M and Greiff L (2001) Unbalanced research. *Trends Pharmacol Sci* **22**: 538–541.

Persson CGA, Erjefält JS, Uller L, Andersson M, Greiff L (2002) Plasma exudation. In: *Asthma and COPD Basic Mechanisms and Clinical Management*, Barnes P, Drazen J, Rennard S and Thomson N (2002) Academic Press, New York, pp. 195–202.

Proud D and Chow CW (2006) Role of viral infections in asthma and chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **35**: 513–518.

Schnackenberg BJ, Jones SM, Pate C, Shank B, Sessions L, Pittman LM, Cornett LE and Kurten RC (2006) The beta-agonist isoproterenol attenuates EGF-stimulated wound closure in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **290**: L485–491.

Tan YR, Qi MM, Qin XQ, Xiang Y, Li X, Wang Y, Qu F, Liu HJ and Zhang JS (2006) Wound repair and proliferation of bronchial epithelial cells enhanced by bombesin receptor subtype 3 activation. *Peptides* **27**: 1852–1858.

Uller L, Rydell-Tormanen K, Persson CG and Erjefält JS (2005) Anti-Fas mAb-induced apoptosis and cytolysis of airway tissue eosinophils aggravates rather than resolves established inflammation. *Respir Res* **6**: 90.

Uller L, Persson CG and Erjefält JS (2006) Resolution of airway disease: removal of inflammatory cells through apoptosis, egression or both? *Trends Pharmacol Sci* **27**: 461–466.

Van Winkle LS, Brown CD, Shimizu JA, Gunderson AD, Evans MJ and Plopper CG (2004) Impaired recovery from naphthalene-induced bronchiolar epithelial injury in mice exposed to aged and diluted sidestream cigarette smoke. *Toxicol Lett* **154**: 1–9.

Wadsworth SJ, Nijmeh HS and Hall IP (2006) Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model. *J Clin Immunol* **26**: 376–387.

Zahm JM, Chevillard M and Puchelle E (1991) Wound repair of human surface respiratory epithelium. *Am J Respir Cell Mol Biol* **5**: 242–248.

5

Integration of Epithelial Ion Transport Activities into Airway Surface Liquid Volume and Ion Composition Regulation

Mark T. Clunes, Peter F. Bove and **Richard C. Boucher**
Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, North Carolina, USA

5.1 Introduction: the role of fluid in airway/alveolar physiology

The alveoli are the primary site of gas exchange in the lung; as such, a wet surface is necessary to facilitate dissolution of gas. The airway, although not involved in gas exchange, conduct air to the alveoli and in the process warm, humidify, and sterilize the air as it passes down the respiratory tree. In the airway, fluid is important, not as a solvent for gas exchange, but as a component of innate lung defence. The fluid lining of the airway supports the flow of secreted mucus up the respiratory tree and in this way acts as the main barrier between inspired pathogens/particulates and the epithelial surface. In addition, a body of evidence is emerging which demonstrates that the physical properties of mucus, e.g., viscoelastic properties and mesh-size of the polymer gel (mucus), are hydration-dependent, and have a major effect upon the ability of mucus to trap and clear bacteria from the airway surfaces (Matsui *et al*., 2005, 2006). Thus, fluid is a crucial element lining all respiratory surfaces from the most proximal nasal epithelium to the most distal alveolar epithelium.

Obviously, regulation of the volume of this fluid is paramount: too much fluid leads to alveolar flooding and impaired gas exchange, while too little fluid leads to airway dehydration and impaired innate lung defence. Little is known about the interplay of fluid transport in the alveolus and the airway. Fluid is likely secreted in the alveolus probably by the type II pneumocytes and absorbed in the airway by the ciliated cells of the surface epithelium. Additional volume is added to airway surfaces by secretions from the submucosal glands

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

present further up the respiratory tree in the larger airway. Lung fluid secretion is a process that starts during early embryo development. Indeed, fluid is crucial developmentally, since it provides a distending pressure that acts as a stimulus for developing air spaces to grow. Paucity of lung epithelial secretion during development leads to reduced alveolarization and thickened alveolar septae at birth. Thus, fluid is a complex and obligate component of alveolar/airway physiology.

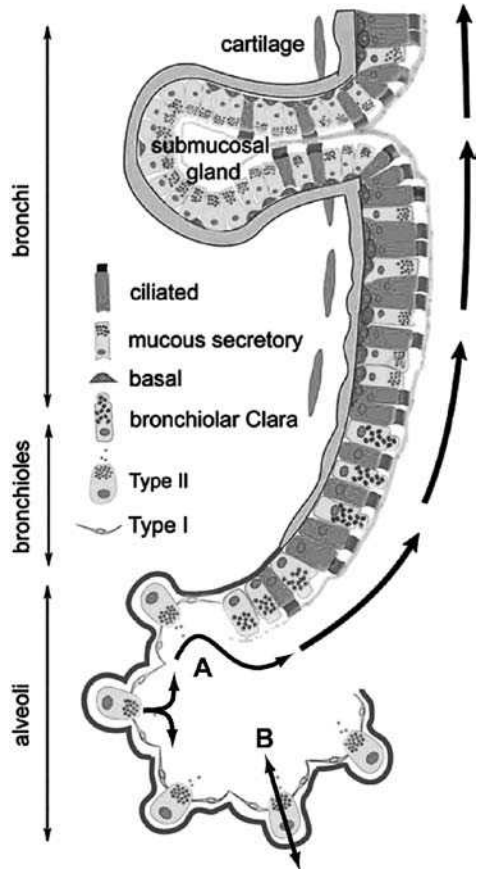


Figure 5.1 Schematic illustration of the human respiratory tract. Ion channels in the apical plasma membrane of airway epithelial cells regulate periciliary fluid depth and mucus hydration. In ciliated cells of the superficial epithelium, the epithelial Na^+ channel (ENaC) mediates Na^+ entry, and Cl^- and water follow through trans and/or paracellular pathways, decreasing airway surface hydration. In contrast, Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) and/or calcium-activated Cl^- channels (CaCC), increases surface hydration. The airway are predominantly absorptive although Cl^- secretion can be stimulated. Submucosal glands are also known to contribute to airway surface liquid in the more proximal airway. In the alveolus, the type II cells are thought to be the primary cells regulating fluid volume on the alveolar surface, with both secretory and absorptive capacity. Little is known about the interface between the alveolus and the terminal bronchioles in terms of fluid handling. Fluid may flow from the alveolus up into the airway (A), or the alveolus may be isolated in terms of its fluid handling, with type II cells balancing secretion and absorption without flow up onto the airway (B)

The surface area of the lung decreases vastly as fluid travels up the respiratory tree. Therefore, as mucus is propelled proximally, fluid volume must be controlled to prevent flooding of the airway (Figure 5.1). However, water transport cannot be achieved directly by biological systems. Cells can, however, transport ions, and those ions can create osmotic gradients that provide the driving force for water flow. The airway epithelium is leaky, and changing the mass of electrolytes on the luminal surface sets up the osmotic gradient required for the flow of water from the interstitial fluid to the lumen. This can occur through

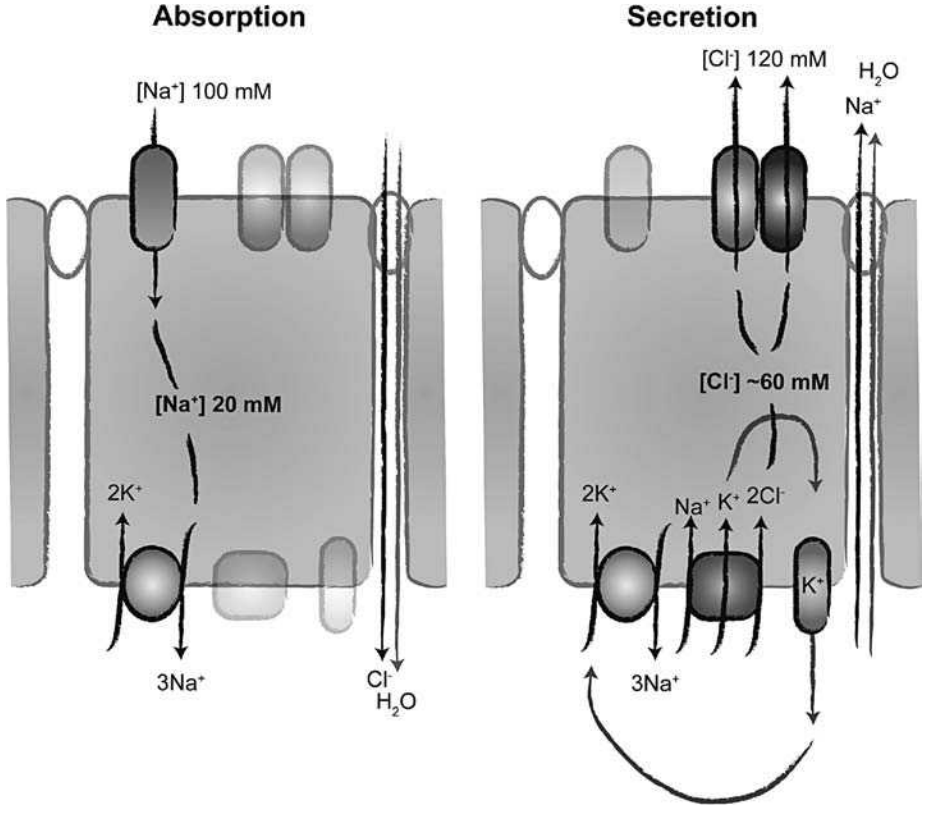


Figure 5.2 Mechanisms of ion transport and fluid secretion in the ciliated cells of the superficial airway epithelium. Water absorption from the ASL is achieved osmotically by increasing the mass of NaCl transported into the basolateral domain. The Na^+-K^+-ATPase maintains a low [Na^+] inside the cell, and provides an electrochemical gradient for Na^+ to flow into the cell through apically located ENaC channels. Na^+ is then pumped into the basolateral space, creating a transepithelial potential difference (PD) (apical negative). The transepithelial PD drives Cl^- from the lumen to the basolateral space via the paracellular route. Thus, increased [NaCl] in the basolateral space forms an osmotic gradient for water flow. In contrast, NaCl transport into the apical space provides the osmotic gradient required for water secretion. Cl^- is accumulated inside the cell by the basolaterally located electroneutral cotransporter. Under basal conditions Cl^- accumulates to $\sim$60 mM and is in electrochemical equilibrium across the apical membrane. Upon stimulation, basolateral K^+ channels open, and ENaC closes, hyperpolarizing the cell and driving Cl^- through apically located channels (CFTR and/or CaCC). The transepithelial PD provides the driving force for Na^+ to flow paracellularly, thus, the osmotic gradient is formed to transport water from the basolateral to the apical space

conductive cellular pathways (i.e. aquaporins) or through the paracellular route. Therefore, ion transport is key for solute flow.

In the airway epithelium, net salt (ion) transport reflects a balance of two main ionic conductances in the apical membrane: a Cl^- conductance dominated by the cystic fibrosis transmembrane conductance regulator (CFTR) and a Na^+ conductance mediated by the epithelial sodium channel (ENaC). CFTR and ENaC are selective ion channels and, as such, conduct ions in response to electrochemical gradients. Vectorial ion movement ultimately depends upon the electrochemical gradients for Na^+ and Cl^- permeation across the apical membrane, generated by pumps and transporters on the basolateral membranes. A summary of the main pumps, transporters, and channels thought to underlie solute transport in airway is shown in Figure 5.2.

5.2 Model of ion and solute transport through airway epithelia

Absorption of Na^+ from the airway surface liquid (ASL) occurs via a 'leak-pump' mechanism. Basolaterally located, the Na^+-K^+-ATPase pump is abundantly expressed and accounts for 20–30 per cent of cellular ATP metabolism under resting conditions. For every two K^+ ions transported into the cell, three Na^+ ions are moved out. This charge disparity sets up an electrical gradient across the cell membrane. It is also largely the Na^+-K^+-ATPase that sets up the inwardly directed Na^+ gradient ($[Na^+]_i \sim 20$ mM vs. $[Na^+]_o$ 100 mM) and outwardly directed K^+ gradient ($[K^+]_i$ 100 mM vs. $[K^+]_o$ 5 mM) observed across epithelial cell membranes. Since there is such a large inwardly directed Na^+ gradient, and a smaller inward electrical gradient (cell interior negative), opening of apical membrane Na^+ channels (ENaC) results in the flow of Na^+ into the cell. Na^+ is then pumped out of the cell to the basolateral interstitial space by the Na^+-K^+-ATPase. This transport process creates a potential difference (PD) across the epithelium (apical side negative). This transepithelial electrical gradient drives anions (which are predominantly Cl^-) out of the apical space, through the paracellular pathway, and into the basolateral space. Thus, NaCl is transported from the apical to the basolateral side of the epithelium and sets up an osmotic gradient for water flow, apical to basolateral.

Secretion is achieved by Cl^- transport from the basolateral to the apical surface of airway epithelia. On the basolateral membrane, the electroneutral cotransporter Na^+-K^+-$2Cl^-$ moves Cl^- into the cell. This step is vital, since Cl^- secretion can be abolished by blocking the cotransporter with bumetanide. Na^+ entering the cell through the cotransporter is pumped back out by the Na^+-K^+-ATPase, while basolateral K^+ channels allow K^+ recycling at the basolateral membrane. Cl^- is thus accumulated inside the cell (40–60 mM), but only reaches electrochemical equilibrium across the apical membrane under resting conditions. The opening of apical Cl^- channels (CFTR or CaCC) must be coordinated with opening of basolateral (and possibly apical) K^+ channels to hyperpolarize the cell interior and provide an electrical driving force for Cl^- to flow from the cell into the ASL. Without the hyperpolarizing effect of opening K^+ channels, there would be no electrical driving force for Cl^- secretion, and indeed, blocking K^+ channels inhibits Cl^- secretion. The transepithelial PD (apical side negative) generated by Cl^- secretion creates an electrical gradient for Na^+ flow through the paracellular path to the apical domain. Thus, the mass of NaCl increases in the ASL and an osmotic gradient is created for water secretion. Water flow, in response

to the osmotic gradients set up by ion transport occurs via cellular conductive pathways, i.e. aquaporins, or via the paracellular route.

Whether or not the secretory and absorptive processes occur in the same cells is still debated. In the gut, secretion is predominantly from the bottom of the crypt, whereas absorption occurs predominantly at the villi tip. Similar spatially distinct models have been suggested for airway. However, to date, the majority of data, e.g., for channel localization and electrophysiological studies, suggest that the superficial airway is predominantly absorptive in nature but can be stimulated to secrete, and that the ciliated cells are the site of both processes (Kreda *et al*., 2005; Rochelle *et al*., 2000; Tarran *et al*., 2006, 2005a).

5.3 Airway histology

The bronchi and bronchioles of human lungs have a pseudostratified epithelium with a variety of different specialized cells. The superficial epithelium of the bronchi / bronchioles is composed mainly of ciliated cells (50–70 per cent surface area), that control mucus flow by ciliary beating, and regulate fluid transport onto the airway surface. Goblet cells are interspersed, and secrete mucins (MUC5AB), which form part of the polymer gel that we know as mucus. In the upper airway and cartilaginous bronchioles, invaginations of the epithelium form a ciliated duct into which the secretions from multiple acini collect. These submucosal glands secrete fluid and mucins (predominantly MUC5B) and importantly, unlike the superficial epithelium, are innervated by cholinergic nerves. Although submucosal glands are known to secrete fluid, their function is not absolutely required, since rat and mouse lungs are known to secrete fluid normally and yet have very few submucosal glands.

5.4 Airway ion secretion

Two main chloride secretory pathways are present in human bronchial epithelial cells, and are often defined by the distinct second messengers that activate each pathway. The predominant pathway is via the cAMP/PKA-mediated Cl^- channel, CFTR. It is known to be the predominant pathway, since absence of this channel, as in cystic fibrosis, leads to reduced fluid on airway surfaces. The CFTR pathway is thought to maintain the basal secretion across the airway, and can be activated by such receptors that increase cAMP, e.g., adenosine receptor A2b (apical), β2 receptor (basolaterally). In addition, an alternative chloride secretory pathway (CaCC), sensitive to increases in free intracellular calcium concentration ($[Ca^{2+}]_i$), is present in airway epithelium. This pathway can be activated by such receptors that signal by increasing $[Ca^{2+}]_i$, e.g., P2Y (apical), cholinergic, bradykinin (basolateral). Together, these two pathways constitute the main conductances present on airway epithelial cell apical membranes that determine anion and water secretion.

5.5 The cystic fibrosis transmembrane conductance regulator

5.5.1 Structure

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a 1480 amino acid polypeptide that forms a two-membrane spanning domain anion channel. On its own,

this polypeptide forms an ATP- and PKA-dependent low-conductance anion channel, typical of that found in the apical membrane of many chloride secreting epithelial cells. It is part of the superfamily of transporters called the ATP-binding cassette transporters (ABC-transporters). Proteins are classified as ABC-transporters, based on a common motif for ATP-binding, the nucleotide binding domain (NBD), of which CFTR has two. The NBDs of ABC-transporters bind ATP, and energy from its subsequent hydrolysis drives the transport of a wide range of substrates across cell membranes. In utilizing the energy from hydrolysis of ATP, substrates can be transported against a concentration gradient. CFTR is unique among the ABC-transporters in that ATP-hydrolysis plays a role in channel function, but that substrate transport is based on the standing electrochemical gradients across the cell membrane, i.e. ATP-hydrolysis regulates CFTR activity but does not drive anions against their electrochemical gradient. While each of the NBDs is able to bind ATP, only NDB2 has ATP-hydrolytic activity, but the exact link between ATP hydrolysis and channel gating remains controversial. Nevertheless, ATP binding is required for channel function.

In addition to the two cytoplasmic NBDs there is an R domain, which is rich in consensus sites for PKA and PKC phosphorylation but displays no definite three-dimensional structure. This regulatory domain is another unique feature amongst the ABC-transporters. The R domain is thought to be involved in driving channel opening, since neither of the NBDs are particularly affected by PKA phosphorylation.

5.5.2 Localization

The predominant location of CFTR in the airway remains controversial. Initial studies, employing both in situ hybridization and immunohistochemistry in human airway demonstrated that CFTR was expressed weakly in the superficial epithelium and the highest expression levels were found in the serous cells of the submucosal gland (Engelhardt *et al*., 1992). This localization was important, since it suggested that submucosal glands were the predominant site for Cl^- secretion and, therefore, the primary site of fluid transport. However, functionally, CFTR is known to be present in well differentiated cultures of human bronchial cells (Tarran *et al*., 2005a), which are almost exclusively composed of ciliated and goblet cells and have a pseudostratifed structure reminiscent of the airway superficial epithelium. A number of studies since have suggested that the ciliated cells of the superficial epithelium do express CFTR and, indeed, the ciliated cells are the primary site of CFTR expression in human airway. One comprehensive study in particular, which examined the respiratory epithelium from the nose to the bronchioles, demonstrated that all ciliated cells, whether on the surface epithelium or in the submucosal gland duct, exhibit CFTR expression in all patients studied. Only half of the patients studied demonstrated CFTR expression in the serous cells of the submucosal glands, suggesting, perhaps, that CFTR expression is lower in these structures (Kreda *et al*., 2005). Since the largest area in contact with airway surface liquid is the airway surface, it makes sense that the superficial epithelium should express channels to regulate solute secretion in airway.

5.6 Calcium-activated chloride channels

Calcium-activated chloride currents capable of stimulating solute transport have been known in airway epithelium for over a decade (Knowles *et al*., 1991), and have generated much

interest, since they represent a secretory pathway still functional in cystic fibrosis airway. However, the molecular basis of these channels remains enigmatic. Unlike CFTR, the CaCC conductance is not regulated by the second messenger cAMP. The calcium activated currents in epithelial cells display some common features, such as the permeability sequence $I^- > Cl^-$ and sensitivity to niflumic acid and 4, 4′-diisothiocyanatostilbene-2, 2′-disulfonic acid (DIDS). These features, along with the complete dependency upon increased intracellular Ca^{2+} for activation of CaCCs, make discrimination of the two main secretory pathways in airway epithelia relatively easy. However, there remains a range of diversities within the described characteristics of CaCC, *e.g.*, range 3–250 pS conductances, differential regulation by calmodulin kinase, and different calcium sensitivities, suggesting that there may be more than one molecular candidate or at least variability in the arrangement of the molecule/s. While no definitive channel has been identified as 'the' CaCC in any epithelial cells, a number of candidates have been proposed.

5.6.1 Bestrophins

Recently, the bestrophin family of chloride channels has been proposed as the putative CaCC underlying the calcium mediated Cl^- secretory response in airway (Kunzelmann *et al.*, 2007). This ~ 70 kDa protein demonstrates a hydropathy profile suggesting a protein with four to six candidate membrane spanning domains. These domains occur within the first 360 amino acids, the region most conserved between bestrophin family members (Tsunenari *et al.*, 2003). Bestrophins have a high sensitivity to Ca^{2+} and demonstrate an EC_{50} for Ca^{2+} of ~ 200 nM, well suited to respond to the changes in $[Ca^{2+}]_i$ generated by receptor activation in epithelial cells. Furthermore, bestrophins 1, 2 and 3 have been detected by RT-PCR in mouse trachea and nasal epithelium, although functional evidence of ATP-mediated chloride secretion and bestrophin 1 protein were only found in trachea. Human lung-derived immortal cell lines expressing bestrophin 1 were found to have ATP-stimulated chloride secretion, whereas those cell lines not expressing bestrophin 1 failed to respond to ATP. This ATP-mediated chloride secretion was DIDS-sensitive and could be abrogated by bestrophin 1 RNAi treatment of cells. In addition, bestrophin 1 localizes to the apical membrane of murine tracheal epithelium (protein not detected in nasal epithelium) and proximal colon (but not distal). An exact functional correlation was found in that ATP-mediated short circuit current could be stimulated in tracheal and proximal colon but neither nasal or distal colon (Barro *et al.*, 2006). Therefore, bestrophins are still in firm contention as the channels underlying CaCC in airway epithelial cells.

5.7 K^+ channels

Although K channels are less well characterized in airway epithelial cells than the apical membrane ion channels for Cl^- and Na^+, they are important nonetheless. K^+ channels largely determine the membrane voltage and provide the driving force for other permeant ions. Driving force is defined by the difference between membrane potential (V_m) and the equilibrium potential of the ion. In normal physiological conditions, the equilibrium potentials (E_x) for Na^+ and Cl^- are $\sim +68$ mV and ~ -30 mV respectively. Since the resting apical membrane potential is in the -30 mV range, a hyperpolarizing effect of opening K^+ channels leads to an increased driving force for Na^+ entry into the cell or an increased driving

force for Cl^- exit, depending on which channels are active in the membrane. Typically, secretagogues stimulate the opening of apical chloride channels and basolateral K^+ channels simultaneously, and inhibition of basolateral K^+ channels leads to inhibition of Cl^- secretion by reducing driving force. Similarly, opening apical membrane Na^+ channels leads to cellular depolarization, and K^+ channel activation offsets that effect.

In airway epithelia, basolateral K^+ conductances can be stimulated by either an increase in $[Ca^{2+}]_i$ or cAMP/PKA. Increased $[Ca^{2+}]_i$ activates $K_{Ca}3.1$(KCNN4/hSK4) K^+ channels which are sensitive to clotrimazole and Ba^{2+} while cAMP/PKA activates the $K_V7.1$ channel that is sensitive to Ba^{2+}, clofilium and chromanol 293B (KCNQ1/K_VLQT1) when associated with the accessory subunit KCNE1 (Mall *et al*., 2000, 2003). However, these channels display low activity under resting conditions, displaying activity only when the epithelia are stimulated to secrete/absorb. The K^+ channels that maintain membrane potential under basal conditions are largely unknown. However, a recent report suggests a role for the Ba^{2+}-insensitive twin-pore domain K^+ channels (Inglis *et al*., 2007).

5.8 Airway ion absorption

5.8.1 Structure of the epithelial Na^+ channel – ENaC

In the airway epithelium, the main channel responsible for sodium, and hence, salt and water absorption, is the epithelial sodium channel ENaC. ENaC is composed of three different subunits (α, β and γ) that share 30 per cent homology at the protein level. Each subunit has a topology predicting two transmembrane domains, short cytoplasmic amino- and carboxy-termini and a large extracellular loop. There is still debate about the stoichiometry of native channel organization, since the α subunit alone can form a Na^+ selective pore, and co-expression of $\alpha\beta$ or $\alpha\gamma$ subunits confer channel activity, albeit well below native channel levels. However, in vitro, co-expression of $\alpha\beta\gamma$ subunits forms a Na^+ selective channel that demonstrates characteristics of the native ENaC conductance, e.g. high Na^+ selectivity, sensitivity to amiloride in nM range and slow gating characteristics. It has been proposed, and there is some consensus, that the native protein is formed with a heterotetrameric structure of $\alpha\beta\alpha\gamma$ subunits (Rossier, 2004). However, other regulatory subunits exist [δ ENaC (Ji *et al*., 2006)], and it is unknown how all of these subunits form as a native protein. What is known, however, is that when $\alpha\beta\gamma$ subunits of ENaC are co-expressed, subunit turnover decreases dramatically, suggesting that the channel is stabilized in the membrane when all subunits interact (Valentijn *et al*., 1998).

5.8.2 Localization

The general pattern of expression of ENaC in respiratory structures of rodents is that of increased ENaC expression in the distal airway, type II cells of the alveolus, and nasal epithelium, with less abundance in the trachea. Interestingly, there is also a difference between lung regions in the ENaC subunit mRNAs that are expressed. In general, in those structures known to be associated with significant amiloride-sensitive ion transport capabilities, e.g., mouse nasal epithelium, rat type II cells, all three ENaC mRNAs were reported. However, in lung and trachea, which display variable amiloride-sensitive current, α ENaC mRNA seems dominant. The bronchus, bronchioles, and alveolar type II cells also all demonstrated a strong presence of $\alpha\beta\gamma$ ENaC mRNA (Rochelle *et al*., 2000). In human lung,

β and γ ENaC has been localized in ciliated cells of the bronchial/bronchiolar surface epithelium, and Clara cells demonstrate detectable amounts of ENaC protein in the apical membrane (Gaillard *et al*., 2000). In situ hybridization has also demonstrated αβγ ENaC mRNAs in the superficial epithelium of airway (Burch *et al*., 1995).

5.8.3 CFTR regulation of ENaC

While ENaC-mediated absorption may be regulated by cAMP raising agonists, CFTR plays a central role in the regulation of ENaC activity in airway epithelia. Patch clamp studies have demonstrated that PKA will activate ENaC in the absence of CFTR, and inhibit it in the presence of CFTR (Stutts *et al*., 1995). ENaC inhibition via cAMP/PKA activation of CFTR is physiologically appropriate, since for effective Cl^- secretion, ENaC must be inhibited to maintain the driving force for Cl^- exit. A tissue-specific interaction between CFTR and ENaC is likely, since in sweat gland duct cells this phenomenon is not apparent. It remains to be resolved how CFTR is capable of inhibiting ENaC activity, e.g., either by a direct interaction or through some unknown accessory protein/pathway.

5.9 Measurement of ion and water transport in airway epithelia

5.9.1 Ussing chamber studies

A classic technique to identify the active ion transport capacities of an epithelium is to perform 'Ussing chamber' experiments. With this technique, freshly excised or cultured cells are placed in hemi-chambers that perfuse each surface of the epithelium independently with a defined solution, typically a Krebs bicarbonate buffer, and the tissues are continually gassed by gas-lift devices. The transepithelial PD (V_t) is measured with macroelectrodes placed near to the apical and serosal surfaces, and V_t is nulled to zero by the passage of current from two silver/silver-Cl^- electrodes placed at an 'infinite' distance from the epithelial surfaces. With this technique, the open circuit V_t is measured, the transepithelial resistance (R_t) calculated from the voltage response to current pulses from Ohm's law, and the current required to null V_t to zero (the short-circuit current, I_{sc}) directly measured or calculated.

The continuously short-circuited approach was developed to identify and quantitate active ion transport by epithelia. This technique was utilized to characterize active ion transport in freshly excised human airway epithelia, both from the upper (nasal) and lower airway (third to sixth generation bronchi) (Knowles *et al*., 1984). As shown in Figure 5.3(A), the dominant active ion transport process under short-circuit conditions is electrogenic, amiloride-sensitive (i.e., ENaC-mediated) Na^+ transport. Little, if any, secretion of Cl^- is measured under these conditions. Studies also identified a small net component of K^+ secretion. This technique also identified the capacity of human airway epithelia to secrete Cl^-. For example, when the apical membrane Na^+ conductance (ENaC) is blocked, the apical membrane potential (V_a) becomes more negative, generating an electrochemical potential for Cl^- secretion. It should be noted that the identification of ion fluxes under short-circuit conditions is perturbed by the fact that short-circuiting an epithelium does modestly change driving forces for ion flow.

Epithelia can also be studied in Ussing chambers under so-called 'open circuit conditions', i.e., when the spontaneous V_t is not nulled. This condition more closely mimics the in vivo

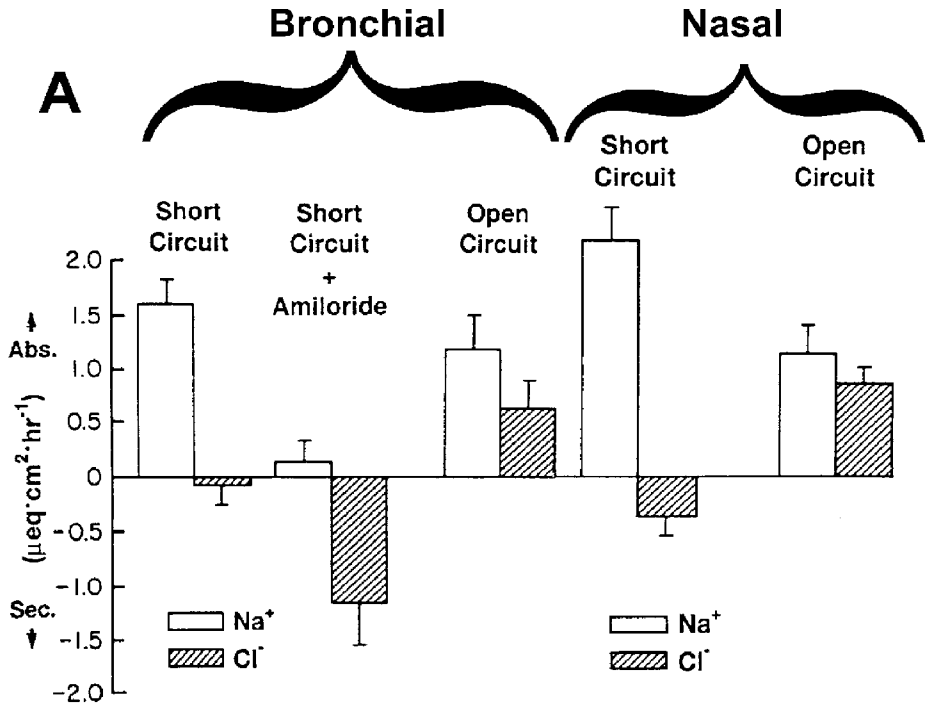


Figure 5.3 Summary of the ion transport processes of nasal and bronchial epithelia under open circuit and short-circuited conditions as measured by radioisotope flux in the Ussing chamber. (A) Under baseline short-circuit conditions, active ion transport is dominated by Na^+ absorption in nasal and bronchial epithelia. Symmetric Cl^- fluxes were observed indicating no net Cl^- transport under baseline conditions. Application of amiloride to block apical ENaC, abolished Na^+ absorption and initiated Cl^- secretion, an effect of cellular hyperpolarization. Under open circuit conditions, the transepithelial PD formed by Na^+ absorption leaves the apical side negative and slows positive charge absorption (Na^+). In addition, the spontaneous transepithelial PD drives Cl^- ions from the lumen resulting in net Cl^- absorption. (B) A summary of the ion transport processes as measured by nasal potential difference in normal and CF patients. Normal nasal PDs are dominated by Na^+ absorption, as shown by their sensitivity to amiloride. Note that CF patients display much higher baseline PDs due to increased Na^+ absorption in the absence of CFTR, which is demonstrated by the large amiloride-sensitive change in PD. Upon perfusion of low chloride, normal epithelia hyperpolarize significantly, indicating a significant contribution to the PD by Cl^-, this effect is absent in CF epithelia due to absence of the main Cl^- conductive pathway, CFTR. Isoprenaline is then added to increase cellular cAMP, and activate CFTR, this effect is maximized in low Cl^- since there is a greater driving force for Cl^- to exit the cell down its concentration gradient into low Cl^-. Again, normal nasal epithelium hyperpolarizes in response to isoprenaline, while CF epithelia display no response. Finally, ATP is perfused to increase intracellular Ca^{2+} concentration via activation of $P2Y_2$ receptors, and initiate calcium-activated chloride currents (CaCC). Both normal and CF epithelia display a robust response to ATP since CaCC are present in CF epithelia, and are unaffected in CF. (A) Reproduced, with permission, from the *American Journal of Respiratory and Critical Care Medicine* 1992, Vol. 150, pp. 271–286. (B) Reproduced with permission from Human Gene Therapy 1995, Vol 6, pp. 445–455

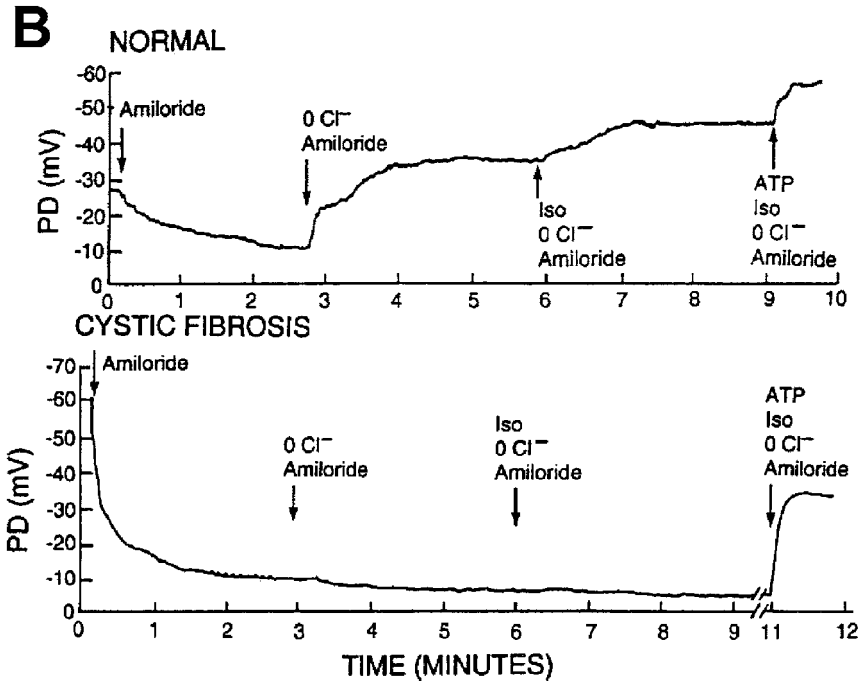


Figure 5.3 (Continued)

situation, and, combined with isotope flux experiments, has yielded important data with respect to the function of human airway epithelia. As shown in Figure 5.3(A), Na^+ absorption persists under open circuit conditions, albeit at a somewhat slower rate, and Cl^- absorption is detected under these conditions (Boucher, 1994). Na^+ absorption is the active component of the net $NaCl^-$ absorptive flux, with Cl^- passively absorbed via the paracellular path to preserve electroneutrality. The application of amiloride blocks Na^+ absorption and induces Cl^- secretion. Under these conditions, active Cl^- secretion occurs via the transcellular path, whereas Na^+ is now 'secreted' passively in response to the electrochemical gradient via the paracellular path. Thus, these studies have highlighted that airway epithelia can both absorb and secrete NaCl, and that, for these functions, a relatively nonselective paracellular path is required for ion transport under open circuit conditions. Direct measures of the permselectivity of the paracellular path have shown that it is, indeed, nonselective (Johnson *et al*., 2004).

5.10 In vivo transepithelial PDs

The transepithelial PD across airway epithelia that is generated by active ion transport can be measured in vivo as well as in vitro. For this technique, a 'ground' electrode is placed in a submucosal compartment, typically under the skin of the forearm, and a flowing solution electrode is placed on the airway epithelial surface, with both electrodes connected via calomel half-cells to a voltmeter. Several manoeuvres can be performed to characterize the

ion transport processes that contribute to the basal PD (Knowles *et al*., 1995). Typically, the absolute magnitude of the PD is a reflection of the rate of Na^+ transport. However, any nonspecific effects on the electrical resistance (R_t) of the epithelial barrier will modify this interpretation. The second manoeuvre, the application of amiloride to the surface, is also employed to estimate the contribution of active Na^+ absorption to the basal PD. However, again, one must be cautious of this interpretation, as amiloride, as described above, not only inhibits Na^+ absorption, but also induces Cl^- secretion. Thus, airway epithelia that manifest a large active Cl^- conductance may exhibit a small amiloride-sensitive PD that does not reflect a small rate of Na^+ transport, but rather, the efficient induction of Cl^- secretion.

Several manoeuvres are utilized to estimate the magnitude of Cl^- conductance in the apical membrane. As noted above, the residual PD after the application of amiloride reflects the capacity of the airway epithelium to secrete Cl^-. This Cl^- secretory capacity is a function of both the magnitude of the apical membrane conductance and the driving force for Cl^- secretion. Since these driving forces may vary in the basal state, a useful manoeuvre to assess the resting Cl^- conductance of the apical membrane is to create a very large artificial driving force for Cl^- secretion by removing all Cl^- from the luminal electrode perfusate. Under these conditions, there is a virtually 'infinite' chemical driving force for Cl^- secretion, and hence, the response of the epithelium to 'low Cl^- solutions' is a reasonable index of the basal Cl^- conductance in the apical membrane.

Finally, manoeuvres are often performed to estimate the relative contribution of the CFTR Cl^- conductance and the Ca^{2+}-activated Cl^- conductance to the resting membrane Cl^- conductance and the regulation of each of these permeabilities, respectively. Typically, to investigate the regulation of CFTR Cl^- conductance, isoproterenol is included in the luminal perfusate to raise cell cAMP and activate CFTR. Thus, the isoproterenol-sensitive PD is a good measure of the regulated CFTR Cl^- conductance in airway epithelia. Conversely, UTP is typically added to the luminal perfusate to activate, via $P2Y_2$ receptors, increases in intracellular Ca^{2+} and, hence, CaCC.

The nasal PD technique has been seminal in identifying the ion transport defects in CF, and it has been a useful tool in identifying and characterizing novel therapeutic agents that may normalize CF ion transport defects. Thus, as shown in Figure 5.3(B), a typical normal nasal PD tracing reveals a basal PD of -30 mV, a PD that is 40 per cent inhibited by amiloride, a large PD response to the 0 Cl^- manoeuvre, and a PD response to both isoproterenol and UTP activation of the CFTR and CaCC conductances, respectively. In contrast, the CF nasal PD tracings exhibit a higher resting basal PD and a larger amiloride-sensitive PD, both reflecting increased Na^+ transport rates, virtually no response to 0 Cl^- manoeuvres, reflecting the absence of resting CFTR function, no response to isoproterenol, reflecting the absence of cAMP-regulated CFTR conductance, and a large response to UTP, reflecting a large CaCC conductance in the apical membrane.

5.11 Volume flow measurements

Epithelial ion transport can modulate the composition of a luminal solution, e.g., reduce the concentration of NaCl (as in sweat), or volume (water) transported across the epithelium. Therefore, studies were performed in which the capacity of airway epithelia to transport volume transepithelially was examined. Miller and colleagues showed that human airway epithelia under baseline conditions absorbed volume, consistent with net Na^+ transport.

Further, volume secretion could be initiated with the application of amiloride (Jiang *et al*., 1993). These studies also suggested that CF airway epithelia hyperabsorb volume, consistent with the raised basal rate of Na^+ transport. Thus, these studies were consistent with airway epithelia as primarily volume-transporting epithelia.

The observation that airway epithelia exhibit volume transport has two implications. First, the prediction is that airway epithelia should be relatively water-permeable. Direct measurements of the hydraulic permeability of the epithelium to water and osmotic water-permeability of airway epithelia have been made in freshly excised tissues and culture preparations (Farinas *et al*., 1997; Matsui *et al*., 2000). All data agree that human airway epithelia are very water-permeable, with the apical membrane being the more permeable of the two barriers. Second, because most epithelia affect isotonic volume transport, the prediction is that the solutions on human airway surfaces are isotonic. Although a matter of some debate in the mid-1990s, most measurements at present suggest that human airway epithelia have an isotonic liquid on their surfaces (Knowles *et al*., 1997; Kotaru *et al*., 2003). The Na^+ concentration is somewhat lower than plasma, i.e., ~ 120 mM, but the K^+ concentration is raised compared to plasma, i.e., ~ 20–25 mM. Thus, sum of the cations $\times 2$ predicts an isotonic solution, consistent with direct measurements of osmolality. The major anions in the ASL are Cl^- and bicarbonate, at approximately the concentrations in plasma.

5.12 Physiologically 'thin film' measurements of ASL volume regulation with confocal microscopy and microelectrodes: studies of normal and CF airway epithelia

Over the past decade, a technique has been developed that measures the physiologic regulation of the ASL compartment under conditions that mimic those observed in vivo. For this technique, well-differentiated cultured cells are utilized (Figure 5.4(A)). These preparations exhibit the capacity to transport ions similarly to freshly excised tissues, have high water permeabilities, secrete mucins, form two layers on their surfaces (periciliary and mucus), and coordinate ciliary activity to effect mucus transport (Matsui *et al*., 1998b). To study ASL volume regulation in these preparations, the ASL is labelled with fluorescent probes, typically Texas red dextran to label the water compartment, and 1-μm beads to label the mucus layer. Microelectrodes are inserted into the thin apical solution ($\sim 7\,\mu$m) with a serosal macroelectrode to measure the transepithelial PD and correlate active ion transport. Interfacing this preparation with a confocal microscope has allowed the dynamic measurement of ASL volume regulation and a dissection of the components of ion transport that mediate these processes.

The typical experiment performed on cultures maintained under standard static tissue culture conditions between confocal measurements is shown in Figure 5.4(B–E) (Tarran *et al*., 2001). In this experiment, a small volume of an ASL mimic (PBS) is added to the apical compartment and the epithelial response to this liquid challenge monitored over time. The normal human airway epithelial culture absorbs the added excess volume and continues to do so until a height of $\sim 7\,\mu$m is reached. At this point, volume absorption stops, and this height is maintained over many hours. A height of $7\,\mu$m appears to be appropriate for key physiologic functions of ASL, as this height allows cilia to extend fully during the ciliary beat

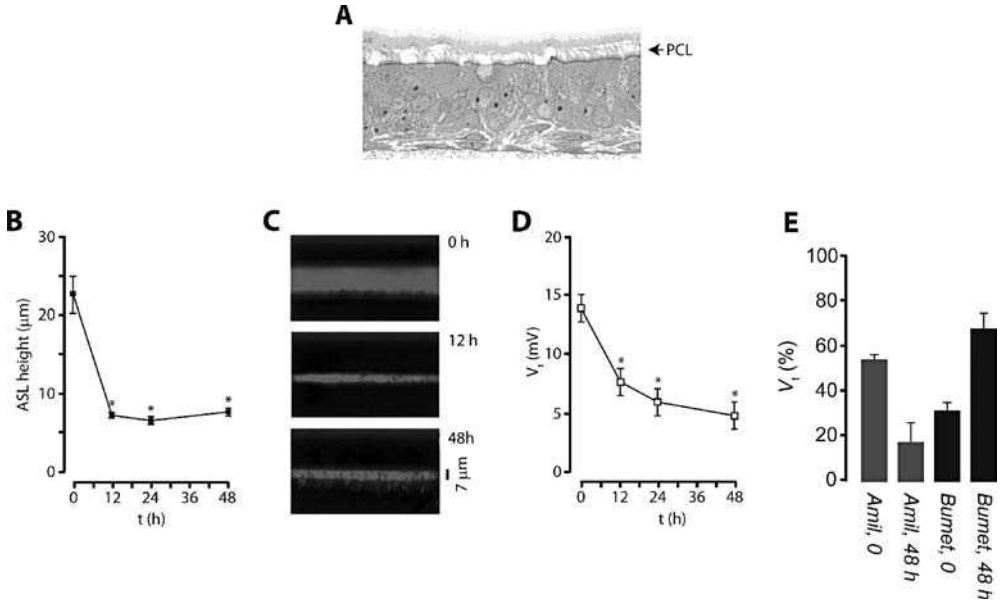


Figure 5.4 Airway surface liquid regulation in cultured human bronchial epithelial cells. (A) Perfluorocarbon OsO_4 fixed human bronchial epithelial cultures display periciliary fluid (PCF) as a clear layer between the cell surface and overlying mucus, cilial shafts can be seen in this layer. The cultures develop as a pseudostratified epithelium with columnar ciliated cells and goblet cells atop the basal cells, reminiscent of native airway epithelium. The PCL can be observed in live cells by inclusion of a cell impermeant fluorescent marker, in this case dextran-labelled Texas red, the height of which is measured using confocal microscopy. (B) and (C) ASL absorption is measured over time after addition of 20 μl of an ASL mimic, phosphate buffered saline (PBS), including dextran-labelled Texas red. ASL volume starts high and over a period of 12 h is absorbed until it reaches a height of ~7 μm, where it is maintained. A 7-μm ASL height allows the cilia to effectively maintain mucociliary transport. (D) and (E) Transepithelial PD is measured after addition of 20 μl of PBS. The PD initially starts high reflecting high rates of amiloride sensitive Na^+ absorption. As ASL is absorbed, overall PD falls and becomes predominantly bumetanide sensitive, reflecting a shift from a predominantly absorbing to a predominantly secreting epithelium. Reproduced, with permission, from the *American Journal of General Physiology*, 2001, 118: 223–236

cycle and move mucus. Evaluation of the bioelectric responses to volume challenge suggests that early, the volume absorption phase is dominated by an amiloride-sensitive absorptive process, whereas later, when volume absorption slows, Na^+ absorption also slows, and Cl^- secretion is induced. When ASL volume steady state is reached, absorption of Na^+ and secretion of Cl^- are balanced (Figure 5.5(A)). Interestingly, this response mimics studies of freshly excised tissues, in which the initial volume stimulus was initiated by gland secretion, followed by absorption of liquid, as measured by cryopreservation techniques (Widdicombe, 2002). Thus, this type of volume regulation appears to be a fundamental process of human airway epithelia.

An important observation made with this technique was that CF airway epithelia, typically missing the CFTR protein in the apical membrane, have a very different volume regulatory response to ASL addition (Matsui *et al*., 1998a; Tarran *et al*., 2005a, 2005b). First, the added

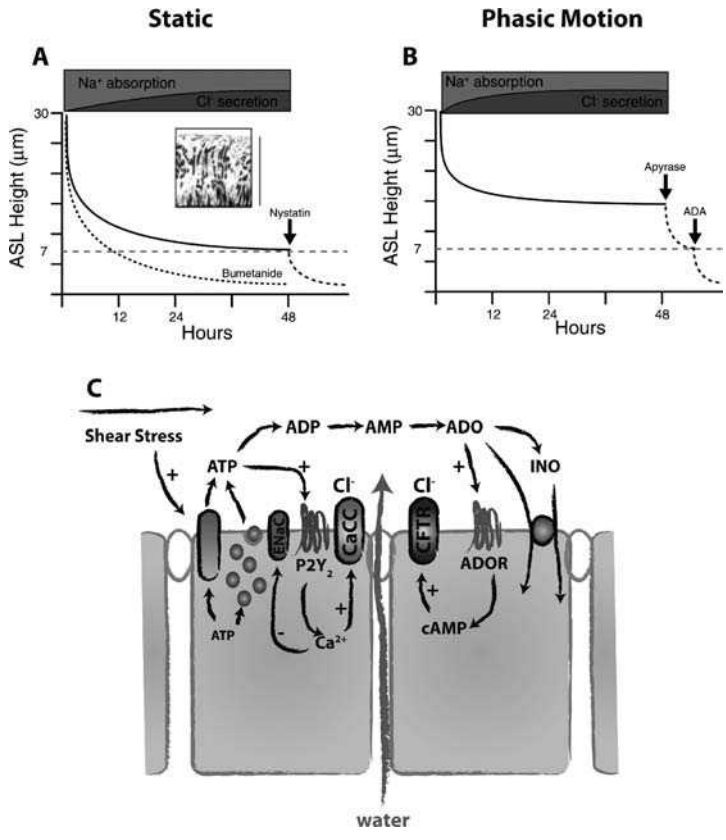


Figure 5.5 Regulation of ASL volume (hydration) by normal human bronchial cells under static and phasic motion culture conditions. Each graph depicts ASL height immediately after deposition of a small volume of PBS followed by confocal measurements. The dashed line in each graph represents the 'normal' ASL height, consistent with effective mucociliary transport. The horizontal bar depicts the relative magnitudes of Na^+ absorption and Cl^- secretion as measured by microelectrode transepithelial PD (post amiloride or post bumetanide for Na^+ and Cl^- transport respectively). The insert in (A), depicts the cilia as observed at 7 μm ASL height, i.e., outstretched and capable of mucociliary transport. In normal airway cultures under static conditions (A) added ASL is reabsorbed until a height of ~ 7 μm is reached, the ability to reach this height reflects the coordinated activity of Na^+ absorption by ENaC and Cl^- secretion via CFTR. After liquid deposition, Na^+ absorption predominates, and as ASL height approaches 7 μm, Na^+ absorption slows and Cl^- secretion increases. If bumetanide is added to inhibit Cl^- secretion or nystatin is added (as an alternative pore for cation entry) to allow unrestrained absorption, then ASL fails to be regulated to 7 μm. Cultures grown under phasic motion (B), display a higher ASL height with two Cl^- secretory components. One component is via calcium-activated Cl^- channels (CaCC), stimulated by ATP release from the epithelium and inhibitable by apyrase (which catalyses ATP breakdown). The second component is via CFTR, which is stimulated by adenosine/A2b/cAMP pathway, and inhibitable by adenosine deaminase (an enzyme that catalyses the breakdown of adenosine). (C) Thus, under resting conditions, secretion is maintained by adenosine/A2b/cAMP activation of CFTR. ATP is rapidly hydrolysed in ASL, producing adenosine, and the low rate of ATP release under resting conditions fails to stimulate CaCC. However, under phasic motion (to produce shear stress), ATP release rate increases and ATP/Ca^{2+} activation of CaCC adds to the secretion by CFTR, while inhibiting Na^+ absorption through ENaC

ASL is absorbed more rapidly, consistent with accelerated net Na^+ absorption. Perhaps more importantly, there is an absence of the appropriate regulation of ASL homeostasis. Specifically, CF airway epithelia absorb all of the liquid from the airway surface, so that cilia cannot extend and beat normally. A corollary is that CF cultures cannot maintain mucus transport under these conditions for a period longer than 24 h.

Bioelectric measurements yielded insights into the mechanisms of this dysfunction. First, Na^+ absorption is not regulated as a function of ASL volume, e.g., Na^+ absorption persists unabated at 48 h despite the fact that virtually all liquid has been removed. Second, CF epithelia cannot initiate Cl^- secretion as the volume of liquid on airway surfaces thins/reduces and approaches 7 μm. Both the failure to regulate ENaC, and the inability to initiate Cl^- secretion, reflect the absence of CFTR function in the CF epithelium.

5.13 The role of physiologic airway shear-stress in ion transport and ASL regulation

A recent improvement in this technique has been the capacity to maintain cells under the phasic motion conditions that reprise the mechanical stresses that are exerted on the airway epithelia in vivo during normal tidal breathing (Button *et al*., 2007; Tarran *et al*., 2005b). Techniques to mimic both the surface airflow during tidal breathing and the transmural compressive stress that occurs during tidal breathing have been developed. Quantitatively different results are observed with respect to ASL volume regulation under these phasic motion conditions. In normal airway epithelia, the height of ASL on airway surfaces increases from ~7 to 14 μm in the steady state. Importantly, in CF airway epithelia, ASL height approximates 7 μm. Correlative mucus transport measurements reveal that mucus transport rates are faster in normals under phasic motion conditions, and restored to measurable levels in CF. Subsequent studies, in which the mucus layer was maintained on airway surfaces, suggested that the extra fluid observed on the normal airway surface during phasic motion conditions is 'stored' in the mucus layer, i.e., it acts as a reservoir (Tarran *et al*., 2001).

These observations raise the intriguing question of what regulates the volume of liquid on airway surfaces in health and disease. This issue is complex and is not fully understood. There appears to be no absolute ASL volume sensor; for example, airway epithelia without cilia appear to exhibit ASL volume regulation quite normally. Rather, it appears likely that ASL volume is not fixed, but can vary in response to local stresses on the surface and disease. For example, it may be more advantageous to have more liquid on airway surfaces during infection and inflammation, increasing the efficiency of mucus transport.

There appear to be at least two pathways that may be important in regulating the ion transport processes that mediate ASL volume homeostasis. First, regulation of the activation state of ENaC (see above) sets the rate of Na^+ transport by the epithelium. It appears that the epithelial Na^+ channel can be activated on airway surfaces by channel-activating proteases (CAP) that convert silent ENaC on the apical membrane into active channels (Vallet *et al*., 1997). Further, it appears that airway epithelia release antiproteases into ASL that can inhibit the activation of CAPs (Bridges *et al*., 2001; Tarran *et al*., 2006; Tong *et al*., 2004). These data have suggested that the antiprotease activity may, in a concentration-dependent manner, regulate the activity of CAPs and hence, ENaC. For example, as ASL volume is reduced, CAP inhibitor concentrations may increase, more effectively inhibit CAP, and slow the rate

of Na^+ absorption. Thus, it is very possible that the CAP/CAP inhibitor system may be critically involved in determining overall rates of Na^+ transport and the sensitivity of the epithelium to Cl^- secretagogues.

The second major system that controls the volume of ASL is the extracellular nucleotide–nucleoside system (Lazarowski *et al*., 2004; Lazarowski and Boucher, 2000). The key concept is that the rate of ATP release from the epithelium, reflecting both a basal rate of release and a shear stress-regulated component, is a major determinant of ASL volume (Figure 5.5 (A–C)) (Tarran *et al*., 2005b). It is important to note that when ATP is released onto the airway surface, there is a complex set of enzymes that will metabolize ATP ultimately to adenosine, which then can be further metabolized to inosine, with both nucleosides being scavenged and taken back up into the epithelial cells by specific transporters (Lazarowski *et al*., 2004). The ratio of ATP to adenosine on airway surfaces will depend on the rate of release and the distribution of the enzymes on the airway surface, both of these processes can be regulated by disease, e.g., inflammation.

ATP itself regulates ASL volume via interactions with a luminally positioned $P2Y_2$ receptor. This receptor is activated by ATP in a concentration-dependent manner to inhibit ENaC via likely at least two mechanisms, by hydrolysis of inner leaflet PIP2 and PKC activation (Ma *et al*., 2002; Yue *et al*., 2002). The inhibition of the apical membrane Na^+ conductance poises the epithelium to secrete Cl^- in response to activation of apical membrane Cl^- conductances. ATP interactions with the $P2Y_2$ receptor activate both CFTR, via a PKC-dependent mechanism, and CaCC, by an IP3-dependent mechanism (Mason *et al*., 1991). Thus, the net effect of ATP activation of $P2Y_2$ receptors is to initiate NaCl and volume secretion.

In parallel, adenosine activates a luminally positioned A_{2b} receptor that is linked to the formation of cAMP in cells (Huang *et al*., 2001). cAMP, via an interaction with PKA, activates the CFTR protein in the apical membrane. CFTR, by as yet unknown mechanisms, can inhibit ENaC itself, and activation of CFTR in the presence of ENaC inhibition generates Cl^- secretion. Thus, normal human airway epithelia exhibit redundant mechanisms for responding to ATP release with secretion, i.e., via ATP/$P2Y_2$-R and adenosine/A_{2b} signalling. Importantly, it is likely that the overall secretion of ASL is ultimately dependent on the nucleotide–nucleoside system.

The physiology of the nucleotide–nucleoside system also explains the differences in physiology for both normal and CF cultures under static phasic motion conditions. For example, under static culture conditions, the rate of ATP release is low ($300\,fmol/cm^2/min$), and the enzyme system on the airway surface converts most ATP to adenosine (ATP concentration $\sim 1\,nM$; adenosine concentration $\sim 100\,nM$). Under these conditions, normal airway epithelia have the capacity to produce sufficient ASL to maintain $7\,\mu m$ of volume on the surface and efficient mucus transport via the ADO-A_{2b}-CFTR axis. In contrast, CF cultures fail to respond to activation of A_{2b} receptors by adenosine due to the absence of CFTR protein in the apical membrane. Thus, they cannot inhibit ENaC and initiate Cl^- secretion as normal cultures do. Under phasic motion conditions, the rate of ATP release is increased $\sim$10-fold. Under these conditions, the ASL ATP concentrations reach ~ 30–$40\,nM$, and the adenosine concentrations, $\sim 200\,nM$. In normal airway epithelia, the $P2Y_2$–R activation of CFTR and CaCC produces the increase in ASL height/volume from $7\,\mu m$ to $14\,\mu m$, as noted above, whereas in CF cultures, the $P2Y_2$–R activation accounts for the increase from $3\,\mu m$ to $\sim 7\,\mu m$ of liquid observed under these conditions.

5.14 Fluid transport across the alveolar epithelium

The distal airway is comprised of two main cell types, the alveolar type I and the alveolar type II cells. Type I cells are large squamous-like cells that constitute most of the internal surface area of the lung, while type II cells are smaller cuboidal cells that cover 2–5 per cent of the alveolar surface (Matthay *et al*., 2005). Since alveolar type II cells can be readily isolated in vitro, they have been studied at length.

Type II cells have been identified as the progenitor cells for the alveolar epithelium, and have been shown to be responsible for restructuring the distal airway after damage to the very susceptible type I cells (Mason, 2006). Type II cells have also been shown to synthesize and secrete surfactant to facilitate proper alveolar expansion (Rooney *et al*., 1994). Moreover, type II cells possess the ability for Na^+ and Cl^- transport, mediating crucial fluid homeostasis within the distal airway. Na^+ uptake occurs on the apical surface of the type II cell, in large part, through an amiloride-sensitive epithelial Na^+ channel (ENaC) (Eaton *et al*., 2004; Jain *et al*., 1999; Yue *et al*., 1995). In the lung, in situ hybridization studies have identified the presence of mRNA for all three subunits of ENaC in vivo and in vitro (Jain *et al*., 1999; Yue *et al*., 1995). Na^+ diffuses through the alveolar cell to the basolateral surface and is pumped into the interstitium by the ouabain-sensitive Na^+, K^+-ATPase pumps (Eaton *et al*., 2004; Factor *et al*., 1998). Ussing chamber studies have identified functional ENaC-mediated vectorial ion transport in vitro across high-resistance rat alveolar type II cell monolayers by measuring short-circuit currents (I_{sc}) (Factor *et al*., 2007). Collectively, this well-regulated process is important for maintaining fluid homeostasis within the alveolus by aiding in the clearance of alveolar edema within intact alveoli.

In addition to understanding the process of the clearance of excess fluid within the distal airway, there have also been studies exploring the transport pathways involved in basal lung Na^+ and water movement. The exact role that ENaC plays, in the absence of agents that are known to stimulate the activity of ENaC, is not clearly understood. However, using RNA interference for α-ENaC, current studies suggested the critical role for ENaC upon β-adrenoceptor stimulation of lung fluid absorption, whereas baseline fluid absorption appeared less dependent on ENaC (Li and Folkesson, 2005).

Although numerous studies have focused on the role of active Na^+ transport as a primary determinant for regulating fluid transport across the distal alveolar epithelium, the involvement of Cl^- transport pathways, mediated by CFTR, and their physiological significance to vectorial fluid transport across the distal lung is still unresolved. Experiments in wild-type mice, and the ex vivo human lung, demonstrated that fluid absorption caused by stimulation was inhibited by glibenclamide, suggesting a role for CFTR-dependent Cl^- absorption (Fang *et al*., 2002, 2006). Moreover, both fluid absorption and Cl^- uptake from the distal airspace were stimulated by β-agonists in wild type, but not in CFTR-mutant (ΔF508) mice.

More recently, alveolar type II cells were identified as one of the major cell types within the distal lung where CFTR may play a role in cAMP-mediated fluid transport, demonstrating that CFTR is expressed in alveolar type II cells, and that the CFTR Cl^- channel contributes to cAMP-regulated fluid transport within the distal airspace of the lung (Fang *et al*., 2002, 2006; Leroy *et al*., 2006). However, a challenge still remains in understanding the relative roles of CFTR-mediated Cl^- secretion or absorption in the alveolus.

Alveolar type I cells, which cover approximately 95 per cent of the alveolar surface, are large, flat cells whose primary function is to mediate gas exchange; however, their

role in ion transport is still unclear. Current thoughts are that type II cells are the main sites of ion transport in the lung, while type I cells provide a passive barrier, rather than an active function. Detailed studies of type I cells have been limited to date, due to the difficulty of maintaining them in cell culture. However, recent studies have demonstrated that type I cells may not only express the machinery for active ion transport, but more importantly, exhibit functional ion transport. For instance, several studies have established a possible contribution of type I cells to vectorial fluid transport. To date, it is established that type I cells express ENaC (Johnson *et al*., 2002), Na^+, K^+-ATPase (Borok *et al*., 2002), and aquaporins, specifically aquaporin 5, an integral membrane protein that facilitates water transport across cell membranes in response to an osmotic gradient (Verkman, 2007). Interestingly, studies have also found functional CFTR in freshly isolated type I cells (Johnson *et al*., 2006), further demonstrating the potential importance of type I cells in maintaining fluid balance.

Important advances have been made in the understanding of the reabsorption of fluid and solutes by the distal alveolar epithelium with characterization of Na^+ and Cl^- transporters under physiological and pathological conditions. Understanding the molecular and biophysical properties of these transporters in vivo, and how these channels are regulated within physiological and pathological environments, is crucial to developing targeted therapeutics.

References

Barro, S. R., Spitzner, M., Schreiber, R., & Kunzelmann, K. Bestrophin 1 enables Ca2+ activated Cl^- conductance in epithelia. *Journal of Biological Chemistry*, 282.

Borok, Z., Liebler, J. M., Lubman, R. L., Foster, M. J., Zhou, B., Li, X., Zabski, S. M., Kim, K. J., & Crandall, E. D. (2002) Na transport proteins are expressed by rat alveolar epithelial type I cells, *American Journal of Physiology – Lung Cell and Molecular Physiology*, **282**, L599–L608.

Boucher, R. C. (1994) Human airway ion transport (Part 1), *American Journal of Respiratory and Critical Care Medicine*, **150**, 271–281.

Bridges, R. J., Newton, B. B., Pilewski, J. M., Devor, D. C., Poll, C. T., & Hall, R. L. (2001) Na^+ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437, *American Journal of Physiology*, **281**, L16–L23.

Burch, L. H., Talbot, C. R., Knowles, M. R., Canessa, C. M., Rossier, B. C., & Boucher, R. C. (1995) Relative expression of the human epithelial Na^+ channel (ENaC) sub-units in normal and cystic fibrosis airways, *American Journal of Physiology*, **269**, C511–C518.

Button, B., Picher, M., & Boucher, R. C. (2007) Differential effects of cyclic and constant stress on ATP release and mucociliary transport by human airway epithelia, *Journal of Physiology (London)*, **580**, 577–592.

Eaton, D. C., Chen, J., Ramosevac, S., Matalon, S., & Jain, L. (2004) Regulation of Na+ channels in lung alveolar type II epithelial cells, *Proceedings of the American Thoracic Society*, **1**, 10–16.

Engelhardt, J. F., Yankaskas, J. R., Ernst, S. A., Yang, Y., Marino, C. R., Boucher, R. C., Cohn, J. A., & Wilson, J. M. (1992) Submucosal glands are the predominant site of CFTR expression in human bronchus, *Nature Genetics*, **2**, 240–247.

Factor, P., Senne, C., Dumasius, V., Ridge, K., Jaffe, H. A., Uhal, B., Gao, Z., & Sznajder, J. I. (1998) Overexpression of the Na+, K+-ATPase alpha1 subunit increases Na+, K+-ATPase function in A549 cells, *American Journal of Respiratory Cell and Molecular Biology*, **18**, 741–749.

Factor, P., Mutlu, G. M., Chen, L., Mohameed, J., Akhmedov, A. T., Meng, F. J., Jilling, T., Lewis, E. R., Johnson, M. D., Xu, A., Kass, D., Martino, J. M., Bellmeyer, A., Albazi, J. S., Emala, C., Lee, H. T., Dobbs, L. G., & Matalon, S. (2007) Adenosine regulation of alveolar fluid clearance, *Proceedings of the National Academy of Sciences of the U.S.A.*, **104**, 4083–4088.

Fang, X., Fukuda, N., Barbry, P., Sartori, C., Verkman, A. S., & Matthay, M. A. (2002) Novel role for CFTR in fluid absorption from the distal airspaces of the lung, *Journal of General Physiology*, **119**, 199–207.

Fang, X., Song, Y., Hirsch, J., Galietta, L. J., Pedemonte, N., Zemans, R. L., Dolganov, G., Verkman, A. S., & Matthay, M. A. (2006) Contribution of CFTR to apical-basolateral fluid transport in cultured human alveolar epithelial type II cells, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **290**, L242–L249.

Farinas, J., Kneen, M., Moore, M., & Verkman, A. S. (1997) Plasma membrane water permeability of cultured cells and epithelia measured by light microscopy with spatial filtering, *Journal of General Physiology*, **110**, 283–296.

Gaillard, D., Hinnrasky, J., Coscoy, S., Hofman, P., Matthay, M. A., Puchelle, E., & Barbry, P. (2000) Early expression of beta- and gamma-subunits of epithelial sodium channel during human airway development, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **278**, L177–L184.

Huang, P., Lazarowski, E. R., Tarran, R., Milgram, S. L., Boucher, R. C., & Stutts, M. J. (2001) Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells, *Proceedings of the National Academy of Sciences of the U.S.A.*, **98**, 14120–14125.

Inglis, S. K., Brown, S. G., Constable, M. J., McTavish, N., Olver, R. E., & Wilson, S. M. (2007) A Ba2+-resistant, acid-sensitive K+ conductance in Na+-absorbing H441 human airway epithelial cells, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **292**, L1304–L1312.

Jain, L., Chen, X. J., Malik, B., Al Khalili, O., & Eaton, D. C. (1999) Antisense oligonucleotides against the alpha-subunit of ENaC decrease lung epithelial cation-channel activity, *American Journal of Physiology*, **276**, L1046–L1051.

Ji, H. L., Su, X. F., Kedar, S., Li, J., Barbry, P., Smith, P. R., Matalon, S., & Benos, D. J. (2006) Delta-subunit confers novel biophysical features to alpha beta gamma-human epithelial sodium channel (ENaC) via a physical interaction, *Journal of Biological Chemistry*, **281**, 8233–8241.

Jiang, C., Finkbeiner, W. E., Widdicombe, J. H., McCray, P. B., Jr, & Miller, S. S. (1993) Altered fluid transport across airway epithelium in cystic fibrosis, *Science*, **262**, 424–427.

Johnson, L. G., Vanhook, M. K., Coyne, C. B., Haykal-Coates, N., & Gavett, S. H. (2004) Safety and efficiency of modulating paracellular permeability to enhance airway epithelial gene transfer *in vivo, Human Gene Therapy*, **14**, 729–747.

Johnson, M. D., Widdicombe, J. H., Allen, L., Barbry, P., & Dobbs, L. G. (2002) Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis, *Proceedings of the National Academy of Sciences of the U.S.A.*, **99**, 1966–1971.

Johnson, M. D., Bao, H. F., Helms, M. N., Chen, X. J., Tigue, Z., Jain, L., Dobbs, L. G., & Eaton, D. C. (2006) Functional ion channels in pulmonary alveolar type I cells support a role for type I cells in lung ion transport, *Proceedings of the National Academy of Sciences of the U.S.A.*, **103**, 4964–4969.

Knowles, M., Murray, G., Shallal, J., Askin, F., Ranga, V., Gatzy, J., & Boucher, R. (1984) Bioelectric properties and ion flow across excised human bronchi, *Journal of Applied Physiology*, **56**, 868–877.

Knowles, M. R., Clarke, L. L., & Boucher, R. C. (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis, *New England Journal of Medicine*, **325**, 533–538.

Knowles, M. R., Paradiso, A. M., & Boucher, R. C. (1995) *In vivo* nasal potential difference: techniques and protocols for assessing efficacy of gene transfer in cystic fibrosis, *Human Gene Therapy*, **6**, 447–457.

Knowles, M. R., Robinson, J. M., Wood, R. E., Pue, C. A., Mentz, W. M., Wager, G. C., Gatzy, J. T., & Boucher, R. C. (1997) Ion composition of airway surface liquid of patients with cystic fibrosis as compared to normal and disease-control subjects, *Journal of Clinical Investigation*, **100**, 2588–2595.

Kotaru, C., Hejal, R. B., Finigan, J. H., Coreno, A. J., Skowronski, M. E., Brianas, L., & McFadden, E. R., Jr. (2003) Desiccation and hypertonicity of the airway surface fluid and thermally induced asthma, *Journal of Applied Physiology*, **94**, 227–233.

Kreda, S. M., Mall, M., Mengos, A., Rochelle, L., Yankaskas, J., Riordan, J. R., & Boucher, R. C. (2005) Characterization of wild-type and {Delta}F508 cystic fibrosis transmembrane regulator in human respiratory epithelia, *Molecular Biology of the Cell*, **16**, 2154–2167.

Kunzelmann, K., Milenkovic, V. M., Spitzner, M., Soria, R. B., & Schreiber, R. (2007) Calcium-dependent chloride conductance in epithelia: is there a contribution by Bestrophin? *Pflugers Archive – European Journal of Physiology*, **454**, 879–889.

Lazarowski, E. R. & Boucher, R. C. (2000) UTP as an extracellular signaling molecule, *News in Physiological Sciences*, **16**, 1–5.

Lazarowski, E. R., Tarran, R., Grubb, B. R., van Heusden, C. A., Okada, S., & Boucher, R. C. (2004) Nucleotide release provides a mechanism for airway surface liquid homeostasis, *Journal of Biological Chemistry*, **279**, 36855–36864.

Leroy, C., Prive, A., Bourret, J. C., Berthiaume, Y., Ferraro, P., & Brochiero, E. (2006) Regulation of ENaC and CFTR expression with K+ channel modulators and effect on fluid absorption across alveolar epithelial cells, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **291**, L1207–L1219.

Li, T. & Folkesson, H. G. (2005) RNA interference for {alpha}ENaC inhibits rat lung fluid absorption in vivo, *American Journal of Physiology*, **290**, L649–L660.

Ma, H. P., Saxena, S., & Warnock, D. G. (2002) Anionic phospholipids regulate native and expressed epithelial sodium channel (ENaC), *Journal of Biological Chemistry*, **277**, 7641–7644.

Mall, M., Wissner, A., Schreiber, R., Kuehr, J., Seydewitz, H. H., Brandis, M., Greger, R., & Kunzelmann, K. (2000) Role of K(V)LQT1 in cyclic adenosine monophosphate-mediated Cl(-) secretion in human airway epithelia, *American Journal of Respiratory Cell and Molecular Biology*, **23**, 283–289.

Mall, M., Gonska, T., Thomas, J., Schreiber, R., Seydewitz, H. H., Kuehr, J., Brandis, M., & Kunzelmann, K. (2003) Modulation of Ca2+-activated Cl- secretion by basolateral K^+ channels in human normal and cystic fibrosis airway epithelia, *Pediatric Research*, **53**, 608–618.

Mason, R. J. (2006) Biology of alveolar type II cells, *Respirology*, **11**, S12–S15.

Mason, S. J., Paradiso, A. M., & Boucher, R. C. (1991) Regulation of transepithelial ion transport and intracellular calcium by extracellular adenosine triphosphate in human normal and cystic fibrosis airway epithelium, *British Journal of Pharmacology*, **103**, 1649–1656.

Matsui, H., Grubb, B. R., Tarran, R., Randell, S. H., Gatzy, J. T., Davis, C. W., & Boucher, R. C. (1998a) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease, *Cell*, **95**, 1005–1015.

Matsui, H., Randell, S. H., Peretti, S. W., Davis, C. W., & Boucher, R. C. (1998b) Coordinated clearance of periciliary liquid and mucus from airway surfaces, *Journal of Clinical Investigation*, **102**, 1125–1131.

Matsui, H., Davis, C. W., Tarran, R., & Boucher, R. C. (2000) Osmotic water permeabilities of cultured, well-differentiated normal and cystic fibrosis airway epithelia, *Journal of Clinical Investigation*, **105**, 1419–1427.

Matsui, H., Verghese, M. W., Kesimer, M., Schwab, U. E., Randell, S. H., Sheehan, J. K., Grubb, B. R., & Boucher, R. C. (2005) Reduced 3-dimensional motility in dehydrated airway mucus prevents neutrophil capture and killing bacteria on airway epithelial surfaces, *Journal of Immunology*, **175**, 1090–1099.

Matsui, H., Wagner, V. E., Hill, D. B., Schwab, U. E., Rogers, T. D., Button, B., Taylor, R. M., II, Superfine, R., Iglewski, B. H., & Boucher, R. C. (2006) A physical linkage between CF airway

surface dehydration and *P. aeruginosa* biofilms, *Proceedings of the National Academy of Sciences of the U.S.A.*, **103**, 18131–18136.

Matthay, M. A., Robriquet, L., & Fang, X. (2005) Alveolar epithelium: role in lung fluid balance and acute lung injury, *Proceedings of the American Thoracic Society*, **2**, 206–213.

Rochelle, L. G., Li, D. C., Ye, H., Talbot, C. R., & Boucher, R. C. (2000) Distribution of ion transport mRNAs throughout murine nose and lung, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **279**, L14–L24.

Rooney, S. A., Young, S. L., & Mendelson, C. R. (1994) Molecular and cellular processing of lung surfactant, *FASEB Journal*, **8**, 957–967.

Rossier, B. C. (2004) The epithelial sodium channel: activation by membrane-bound serine proteases, *Proceedings of the American Thoracic Society*, **1**, 4–9.

Stutts, M. J., Canessa, C. M., Olsen, J. C., Hamrick, M., Cohn, J. A., Rossier, B. C., & Boucher, R. C. (1995) CFTR as a cAMP-dependent regulator of sodium channels, *Science*, **269**, 847–850.

Tarran, R., Grubb, B. R., Gatzy, J. T., Davis, C. W., & Boucher, R. C. (2001) The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition, *Journal of General Physiology*, **118**, 223–236.

Tarran, R., Button, B., & Boucher, R. C. (2005a) Regulation of normal and cystic fibrosis airway surface liquid volume by phasic shear stress, *Annual Review of Physiology*, **68**, 543–561.

Tarran, R., Button, B., Picher, M., Paradiso, A. M., Ribeiro, C. M., Lazarowski, E. R., Zhang, L., Collins, P. L., Pickles, R. J., Fredberg, J. J., & Boucher, R. C. (2005b) Normal and cystic fibrosis airway surface liquid homeostasis: the effects of phasic shear stress and viral infections, *Journal of Biological Chemistry*, **280**, 35751–35759.

Tarran, R., Trout, L., Donaldson, S. H., & Boucher, R. C. (2006) Soluble mediators, not cilia, determine airway surface liquid volume in normal and cystic fibrosis superficial airway epithelia, *Journal of General Physiology*, **127**, 591–604.

Tong, Z., Illek, B., Bhagwandin, V. J., Verghese, G. M., & Caughey, G. H. (2004) Prostasin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line, *American Journal of Physiology*, **287**, L928–L935.

Tsunenari, T., Sun, H., Williams, J., Cahill, H., Smallwood, P., Yau, K. W., & Nathans, J. (2003) Structure–function analysis of the bestrophin family of anion channels, *Journal of Biological Chemistry*, **278**, 41114–41125.

Valentijn, J. A., Fyfe, G. K., & Canessa, C. M. (1998) Biosynthesis and processing of epithelial sodium channels in *Xenopus oocytes, Journal of Biological Chemistry*, **273**, 30344–30351.

Vallet, V., Chraibi, A., Gaeggeler, H. P., Horisberger, J. D., & Rossier, B. C. (1997) An epithelial serine protease activates the amiloride-sensitive sodium channel, *Nature*, **389**, 607–610.

Verkman, A. S. (2007) Role of aquaporins in lung liquid physiology. *Respiratory Physiology and Neurobiology*, in press. DOI: 10.1016/j.resp.2007.02.012.

Widdicombe, J. H. (2002) Volume of airway surface liquid in health and disease, *American Journal of Respiratory and Critical Care Medicine*, **165**, 1566.

Yue, G., Russell, W. J., Benos, D. J., Jackson, R. M., Olman, M. A., & Matalon, S. (1995) Increased expression and activity of sodium channels in alveolar type II cells of hyperoxic rats, *Proceedings of the National Academy of Sciences of the U.S.A.*, **92**, 8418–8422.

Yue, G., Malik, B., Yue, G., & Eaton, D. C. (2002) Phosphatidylinositol 4,5-bisphosphate (PIP2) stimulates epithelial sodium channel activity in A6 cells, *Journal of Biological Chemistry*, **277**, 11965–11969.

6

Structure and Function of Cilia

Andreas Schmid and **Matthias Salathe**
Division of Pulmonary and Critical Care Medicine, University of Miami Miller School of Medicine, Miami, Florida, USA

6.1 Introduction

In general, the mucociliary apparatus serves several important functions in the airway. The major function is to provide a mechanical barrier for trapping particulates in the surface liquid covering the airway epithelium and clearing them from the tracheobronchial tree by ciliary action, a mechanism called mucociliary clearance. Cilia are the motors for this transport while mucus (see Chapter 7) serves as the transport vehicle for foreign substances.

Cilia are restricted to the conductive airway proximal of the respiratory bronchioles. While the size of the total alveolar surface of a normal human lung is approximately $85\,m^2$, the ciliated surface measures only about $0.15\,m^2$. This ciliated area, however, is responsible for propelling all particles deposited onto the airway surface to the pharynx. Cilia beat in a low-viscosity, aqueous environment called the periciliary liquid layer. This layer is covered by mucus, which binds and entraps deposited particles for transportation out of the airway. For mucociliary transport to be effective, several epithelial functions need to properly interact with each other, including epithelial water and ion transport (see Chapter 5), mucin secretion (see Chapter 7), and ciliary action. If mucociliary clearance fails, airway and lung disease ensue as illustrated by diseases such as chronic bronchitis, cystic fibrosis or primary ciliary dyskinesia. This chapter will focus on the motor aspect of this transport, namely the cilia, including their structure and function as well as the relation of their functional failure to diseases.

6.2 Structure

Each motile human cilium is approximately 6–7 μm long and 0.2–0.3 μm in diameter. Structurally, the cilium consists of a microtubular axoneme surrounded by a membrane that is in continuation with the plasma membrane of the cell but seems to contain specific proteins otherwise not found in the apical membrane and vice versa. The axoneme is

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

made up of microtubules, dynein arms, radial spokes and interdoublet links (Figure 6.1). The ultrastructure of axonemes has been preserved throughout evolution and has been well characterized by electron microscopy. In addition, recent advances in proteomics have enabled delineation of a catalogue of ciliary proteins. Pazour *et al*. identified 360 proteins with high confidence and an additional 292 proteins with moderate confidence in flagella of *Chlamydomonas rheinhardii* (Pazour *et al*., 2005), while Ostrowski *et al*. identified more than 200 proteins and over 200 expressed sequence tags (ESTs) in human axonemes (Ostrowski *et al*., 2002). Such catalogues make clear that the axoneme is an incredibly complex structure and remind us that we are still far from understanding its structure–function relationship completely. This section can only provide a brief overview of the complexity of the ciliary structure and function.

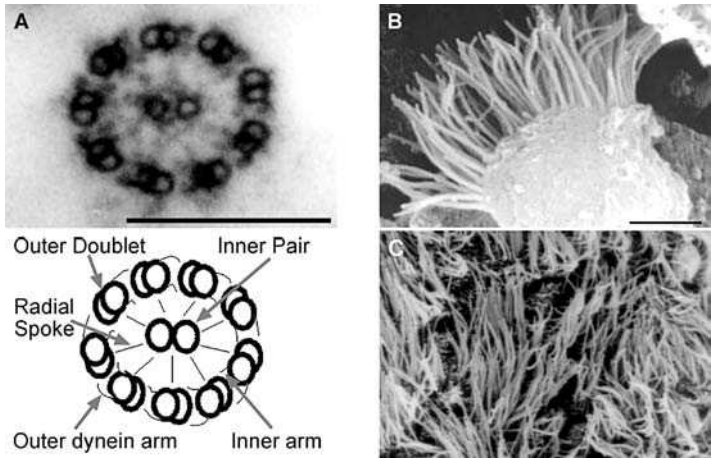


Figure 6.1 Structure of respiratory cilia. (A) Transverse section by transmission electron microscopy through an ovine cilium with a schematic and explanations below. Bar is 0.3 μm. (B) Single airway epithelial cell by scanning electron microscopy. Bar is 5 μm. (C) Surface of ciliated ovine airway epithelium by scanning electron microscopy. Magnification × 2000

6.2.1 Membrane

Until recently, there has been little information available on the composition and function of human airway ciliary membrane proteins. In the proteomic analysis by Pazour *et al*. mentioned above, 39 of the identified 360 proteins were known to be membrane components (Pazour *et al*., 2005). The identified proteins included six ion pumps or channels, three plasma membrane Ca^{2+}-ATPases and four closely related proteins that have 8 to 12 transmembrane helixes, and a domain that is a sensory motif involved in detecting such diverse stimuli as light, oxygen, redox state and small ligands (Taylor and Zhulin, 1999). Identification of the latter proteins suggests that the ciliary membrane performs an important sensory and signal transduction function. This is a relatively new concept for motile cilia, even though it has been known that cilia can increase their beating force when adjusting to the changes in the outside environment such as increasing viscosity of the periciliary fluid layer (Johnson *et al*., 1991). Thus, signalling molecules could be expressed on the ciliary membrane. In support of this hypothesis, recent publications describe expression of the tyrosine kinases Tie-1 and Tie-2

(Teilmann and Christensen, 2005), the transient receptor potential vaniloid 4 (TRPV4) cation channel and polycystin-1 and polycystin-2 (Teilmann *et al*., 2005) on motile cilia of the oviduct in mice and humans. TRPV4 has been shown to regulate ciliary beat frequency (CBF) in hamster oviduct in response to increasing viscosity by allowing Ca^{2+} influx (Andrade *et al*., 2005). Thus, an increasing number of publications suggest that the ciliary membrane is important in regulating CBF and beating force. Other functions may also be attributed to the ciliary membrane as a recent publication reveals expression of the organic cation/carnitine transporter N2 (OCTN2) at this location (Horvath *et al*., 2006).

6.2.2 Axoneme

The axoneme is the detergent-resistant, membrane-stripped structure of the cilium. The ultrastructure of motile axonemes is typically described as a $9 + 2$ arrangement of doublet microtubules as seen in transmission electron microscopy studies (Figure 6.1). But besides microtubules, cilia also contain a large number of other structural elements including inner and outer dynein arms (the motors of movement), radial and circumferential spokes and interdoublet links (Satir and Sleigh, 1990).

The outer microtubular doublets consist of an A and B subfibre, both assembled from α and β tubulin heterodimers (Nogales *et al*., 1999) with the polymerizing (+) end at the ciliary tip. There, the doublets simplify to single tubules (subfibre A) which insert into a disc that usually forms the cytoplasmic surface of a transmembrane complex, called the ciliary crown (Satir and Sleigh, 1990). At the base, the axoneme ends on a centriole, called a basal body, where cytoplasmic microtubules also attach, thereby stabilizing the ciliary machinery on the cytoskeleton. There seems to be a 'gate' at the ciliary base, controlling what is allowed to enter the cilium. While small molecules can likely enter the cilium by diffusion (calcium and cAMP for instance), others need to be transported via intraflagellar transport, a feature immensely important for proper assembly and function of cilia (Scholey and Anderson, 2006; Rosenbaum and Witman, 2002).

Radial and circumferential linkages integrate the individual microtubules into a functioning axoneme (Satir and Sleigh, 1990). The T-shaped radial spokes connect the doublet microtubules to the central pair complex. They usually are arranged in groups of three along subfibre A and extend the entire ciliary length with a 96-nm period (McEwen *et al*., 1986). Studies of *Chlamydomonas* revealed that radial spokes contain at least 23 distinct polypeptides (Yang *et al*., 2001; Piperno *et al*., 1981), with a combined molecular mass of approximately 1200 kDa. Many of these identified proteins are predicted to contain domains associated with signal transduction, including Ca^{2+}-, AKAP- and nucleotide-binding domains (Yang *et al*., 2006). These studies show that radial spokes are far more than just connectors between microtubules, at least in *Chlamydomonas*, but likely also in mammalian cilia (even though the regulation of beating is somewhat different between the two).

Interdoublet links were termed nexins (Stephens, 1970). Nexins are arranged along the doublets every 86 nm between adjacent subfibres A and B (Warner, 1976). Recent studies using cryoelectron tomography (Nicastro *et al*., 2006) interpret nexin as a major part of the dynein regulatory complex (see below) with connection to the A subfibre, close to the attachment of the second radial spoke, and the adjacent B subfibre. This arrangement suggests that nexins may mediate regulatory signals between radial spokes and inner and outer dynein arms. The initially predicted elastic character of the protein (Warner, 1976) is supported by its zigzag structure (Nicastro *et al*., 2006).

Dyneins are the force-producing molecular motors that cause the doublet microtubules to slide with respect to one another (Satir and Christensen, 2006). Outer and inner dynein arms are attached to the dynein regulatory complex (Nicastro *et al*., 2006). All outer dynein arms have the same structure in electron microscopy studies. In mammalian tracheal cilia, the outer dynein arm is a two-headed bouquet-like molecule with a molecular size of 1–2 million Da (Hastie *et al*., 1988). Each head contains a heavy chain ATPase of 400 000–500 000 Da. During ciliary beating, these dynein heavy chains interact with adjacent microtubules and move the microtubules relative to each other. According to studies in *Chlamydomonas*, the outer dynein arm is the frequency-regulating center of the cilium (Brokaw and Kamiya, 1987), and some limited studies in patients who suffer from respiratory illnesses due to missing outer dynein arms seem to confirm this notion (Chilvers *et al*., 2003). Further sophisticated analysis of beating patterns are now under way to evaluate whether these data can be confirmed in a large population (C. William Davis, personal communication).

Inner dynein arms are structurally and functionally more complex than outer dynein arms. In *Chlamydomonas*, at least eight different inner-arm dynein heavy chains are organized with various dynein intermediate and light chains into seven distinct complexes: one two-headed dynein and six single-headed isoforms (Kamiya, 2002). By electron microscopy one double-headed and five single-headed inner dynein arm complexes could be shown (Nicastro *et al*., 2006).

As already exemplified by the assembly of nexins and radial spokes, cilia are built as repetitive 'modules' along the ciliary axis. The basic module is 96 nm in length, consisting of four outer dynein arms, three inner dynein arms, three radial spokes and one pair of interdoublet links (Satir and Sleigh, 1990).

6.3 Function

6.3.1 Beat pattern of single cilia

After an effective stroke in the direction of mucus transport, the cilium goes through a recovery stroke by swinging almost 180° horizontally backward more closely to the cell surface and in a plane perpendicular to that surface. Extended almost to full length, the effective stroke reaches a maximal velocity of 1 mm s^{-1} at the ciliary tip describing an arc of approximately 110°, thereby propelling the mucus towards the pharynx. The effective stroke is approximately two to three times faster than the recovery stroke (Sanderson and Dirksen, 1985). These early studies suggested that the cilium rests shortly after completing the effective stroke before resuming motion into the recovery and effective strokes. Recent data, however, have started to cast doubt on the resting phase of the cilium (P. Sears and C. William Davis, personal communication).

Dyneins are the motor molecules and they produce sliding of the microtubules relative to each other. Since the motor activity is restricted to a single polarity, the movement can only go in one direction. In order to achieve three-dimensional motion, some asynchrony of the arm activity must therefore be present. A hypothesis for how this asynchrony may work was provided by the switch point theory (Satir and Sleigh, 1990). This hypothesis states that half of the doublets of the axoneme have active arms when the axoneme is moving through its effective stroke and that the other half has active arms during the return stoke. When a switch is blocked, the cilia will come to rest in one specific position, no matter where in the

beat cycle the block is applied. Blocking the second switch will lead to ciliary arrest in a second position (Satir and Sleigh, 1990). As shown in mussel gill cilia, one arrest position is near the beginning of the effective stroke and the second is near the beginning of the recovery stroke (Wais-Steider and Satir, 1979). The overall movement, however, is more complicated and more work is needed to really understand how dynein arms can bring about a complex motion in their arrangement in the axoneme.

6.3.2 Coordination of ciliary beat

Cilia are oriented to beat in the same, or at least similar, direction within a plane roughly perpendicular to the epithelial surface. How cilia align during development to beat in the same direction remains a mystery. The coordination between beating cilia gives rise to the metachronal wave. The wavelength of a metachronal wave has been measured to be around 5–9 μm. The wave propagation has been reported at different angles (likely depending on the length of the measurement field) and up to 125° clockwise to the direction of the effective stroke (Gheber and Priel, 1994; Wong *et al*., 1993). The mechanisms of how cilia are coordinated to create these waves are not well understood. Cilia on single cells seem to beat together (personal unpublished observations and Gheber and Priel, 1989), but this is not necessarily true for cilia on different cells if the cilia of these cells are farther apart than about 10 μm (Gheber and Priel, 1989). These findings thus imply that the close spatial relationship between cilia is important for their coordination. Furthermore, the environment in which cilia beat consists at least in part of fluid; thus, significant hydrodynamic forces must exist between beating cilia. These hydrodynamic interactions are believed to be the most important factor for ciliary coordination on epithelial surfaces (Gheber *et al*., 1998) and may explain why the lengths of metachronal waves are limited (Gheber and Priel, 1989; Sanderson and Sleigh, 1981a).

6.3.3 Regulation of CBF

CBF changes are modulated by changes in the phosphorylation state of ciliary targets, the intracellular calcium concentration ($[Ca^{2+}]_i$), intracellular pH (pH_i) as well as changes in $[HCO_3^-/CO_2]_i$, independent of pH changes (Figures 6.2 and 6.3).

cAMP-dependent modulation of CBF

It is well accepted that axonemal beating can be stimulated by cAMP in different mammalian species (Sanderson and Dirksen, 1989; Salathe *et al*., 1993a; Wyatt *et al*., 1998, 2005; Di Benedetto *et al*., 1991). Sources of cAMP in airway epithelial cells are usually thought to be the G-protein coupled, transmembrane adenylyl cyclases (tmAC). However, the presence of soluble adenylyl cyclase (sAC) has been described as well (Schmid *et al*., 2005) and cAMP from this source could be important for ciliary beating. Therefore, the cell has multiple possibilities to regulate CBF via increases in cAMP: through stimulation of G-protein-coupled receptors (e.g., β2- or A2b receptors, Salathe, 2002; Morse *et al*., 2001), direct calcium activation of tmACs, and activation of sAC by CO_2/HCO_3^- (Schmid *et al*., 2005).

Phosphodiesterases are strategically localized around the areas where cAMP is produced to create micro-domains with high concentrations of cAMP that is not allowed to diffuse freely throughout the cell. It seems that the effects of cAMP on the axoneme and thus on

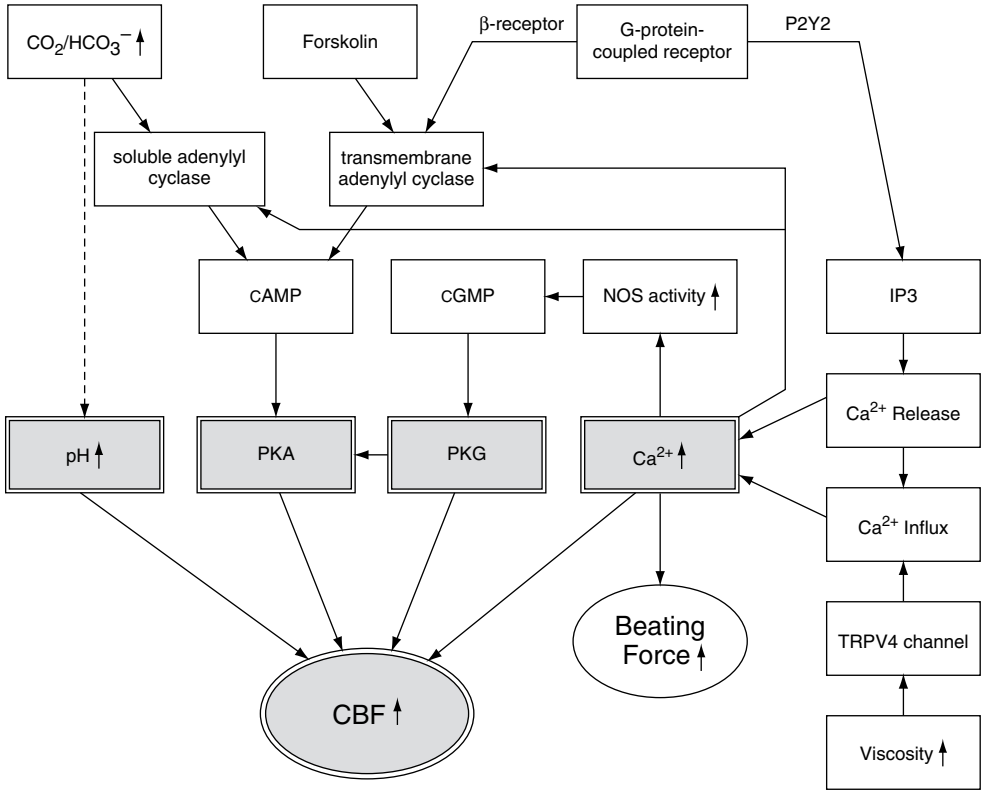


Figure 6.2 Diagram of signalling pathways that increase CBF. See text for details

ciliary beating are mainly mediated by protein kinase A (PKA), which is phosphorylating an outer arm dynein light chain. This target was originally described in *Paramecium* but it is also found in several mammalian cilia including the human airway axoneme.

Calcium

Whereas cAMP-dependent regulation of CBF seems to be similar between unicellular organisms and mammals, the regulation of CBF by Ca^{2+} is, at least in some aspects, different. In mammals, elevation of $[Ca^{2+}]_i$ is always associated with an increase of CBF. The regulation of CBF by Ca^{2+} occurs within one beat cycle and only small changes in $[Ca^{2+}]_i$ are needed to change CBF (Salathe and Bookman, 1999; Lansley and Sanderson, 1999; Zhang and Sanderson, 2003a). The mechanism of the Ca^{2+}-mediated regulation of CBF is debated. Even though some reports indicated the involvement of kinases in the CBF response to initial Ca^{2+} changes, others have provided clear evidence that Ca^{2+} acts directly on a ciliary target (Salathe and Bookman, 1999; Zhang and Sanderson, 2003a). It is also clear, however, that transient Ca^{2+} increases can activate additional pathways that have an effect on CBF such as cAMP and cGMP pathways (Zhang and Sanderson, 2003a, 2003b; Lieb *et al*., 2002). Whether or not the initial Ca^{2+} response requires a baseline level of cyclic nucleotides (Ma *et al*., 2002) remains unclear.

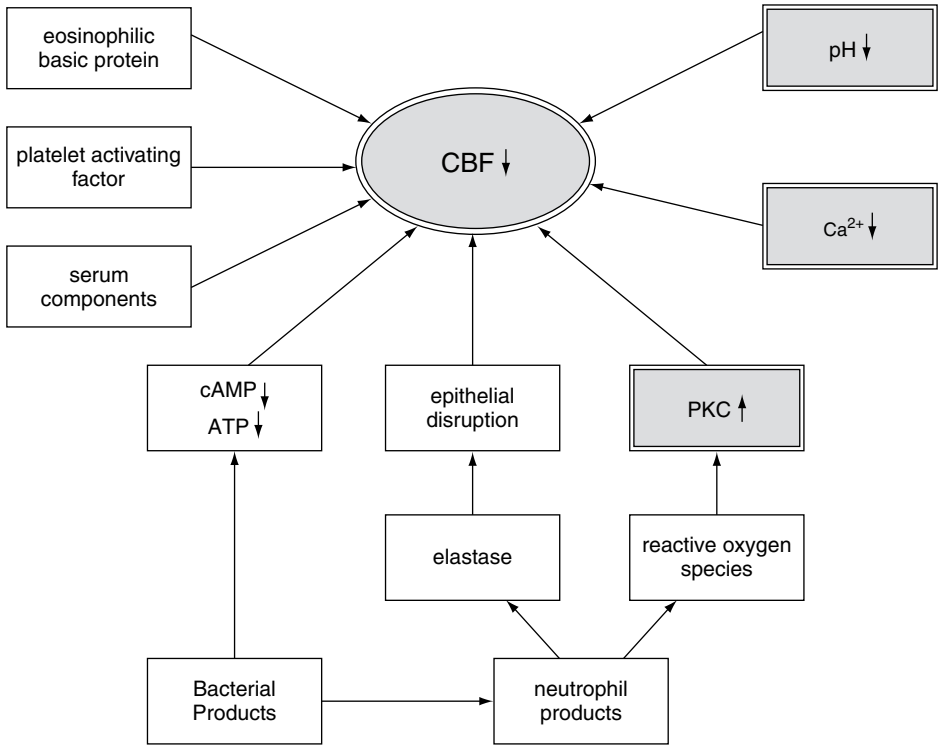


Figure 6.3 Diagram of signalling pathways that decrease CBF. See text for details

Cyclic GMP modulation of CBF

cGMP is produced by activation of either soluble or membrane-bound guanylyl cyclase. The membrane-bound form is stimulated by c-type natriuretic peptide and atrial natriuretic peptide and the soluble form by nitric oxide (Padayatti *et al*., 2004). cGMP is described to elevate CBF in mammalian airway cells, involving the activation of PKG (Wyatt *et al*., 1998, 2005; Geary *et al*., 1995). In bovine axonemes, CBF increases are similar upon stimulation of comparable concentrations of cAMP and cGMP, but the highest stimulation was achieved with a combination of cAMP and cGMP (Wyatt *et al*., 2005). cGMP has been shown to regulate CBF in rabbit airway in both a Ca^{2+}-dependent and an independent manner. Since the cGMP-PKG signalling pathway is not required for rapid, calcium-dependent increases in CBF, cGMP seems to act independently of Ca^{2+} (Zhang and Sanderson, 2003b). However, calcium can also activate the cGMP-dependent pathway (Zhang and Sanderson, 2003b).

pH_i-dependent modulation of CBF

CBF in human airway epithelial cells is also regulated directly by pH_i. Changes in extracellular pH per se (without influencing pH_i) seems to have only minimal effects on CBF, unless the pH becomes extreme and likely changes pH_i (Clary-Meinesz *et al*., 1998; Kienast *et al*., 1994). On the other hand, small changes in pH_i have profound effects on CBF

(Sutto *et al*., 2004). Alkalization increases CBF whereas acidification decreases CBF and these changes are not related to PKA activation/inhibition or influences on other protein kinases and phosphatases (Sutto *et al*., 2004). These findings therefore support a direct effect of pH_i on CBF. In favour of this hypothesis, human spermatozoa lacking outer dynein arms failed to exhibit higher beat frequency during mild alkalization in contrast to normal spermatozoa (Keskes *et al*., 1998).

6.4 Ciliary dysfunction associated with disease

6.4.1 Primary ciliary dyskinesia (PCD)

Motile cilia play a crucial role in clearing mucus and debris from the airway. If cilia are dysfunctional, airway disease ensues. This is clearly demonstrated in patients suffering from primary ciliary dyskinesia or PCD (e.g., Mitchison *et al*., 2006; Moller *et al*., 2006). A report of a patient with symptoms of bronchiectasis and *situs inversus* one hundred years ago is likely the first account of PCD (Siewert, 1904). Kartagener added chronic sinusitis to the syndrome that was then named after him. About thirty years ago, abnormalities in the ultrastructural composition of motile cilia were finally recognized by Afzelius as the cause of PCD (Afzelius, 1976). Many clinical features of PCD are thus related to impaired mucociliary clearance and include rhinitis, sinusitis, otitis media, and chronic productive cough. The loss of normal mucociliary function leads to bronchiectasis.

There is considerable heterogeneity of dynein arm abnormalities in these patients. A recent study analysing the different defects reported that 43 per cent of PCD patients have outer dynein arm defects, 29 per cent have inner dynein arm defects, and 24 per cent have defects of both arms (Noone *et al*., 2004). However, anomalies of the central microtubular pairs, radial spokes, or nexin links and abnormal alignment of the beating plane can also cause abnormal ciliary beating and thus lead to PCD.

Genetic approaches have elucidated at least some of the heterogeneous molecular defects underlying PCD (reviewed in Zariwala *et al*., 2006) by focusing on genetic linkage analysis and candidate gene analysis. Linkage analysis has identified several PCD loci, including *DNAH5* on chromosome 5p15, *CILD2* on 19q, and additional loci on 16p12 and 15q13–15. Selection of candidate genes for mutational analysis has also proved successful with identification of mutations in *DNAI1* on chromosome 9p13-p21 and *DNAH11* on 7p15.

The reasons for *situs inversus totalis* in patients with PCD has been at least partially elucidated. The cloning of an axonemal dynein heavy-chain gene, left/right-dynein, that was mutated in a strain of mice with a 50 per cent incidence of *situs inversus*, was the first clear indication that *situs inversus* was related to a ciliary defect (Supp *et al*., 1997). Then, cilia were found in the embryonic node at the time of left–right asymmetry determination and these cilia were motile despite their '$9+0$' ultrastructure. This motility creates a directional flow across the embryonic node that seems to determine left–right asymmetry (McGrath *et al*., 2003; Hirokawa *et al*., 2006; Nonaka *et al*., 1998).

6.4.2 Other airway diseases associated with abnormal ciliary function

Bacteria, bacterial products, and viruses

Hemophilus influenzae, a bacterium commonly encountered in chronic bronchitis, can induce epithelial cell damage (Dowling *et al*., 1998) and ciliary dysfunction (Wilson *et al*., 1985).

In addition, bacterial products from *Pseudomonas aeruginosa* such as hydroxyphenazine, pyocyanin, and a rhamnolipid as well as bacterial culture supernatants have been shown to decrease CBF (Wilson *et al*., 1985, 1987, 1988; Jackowski *et al*., 1991; Hingley *et al*., 1986; Kanthakumar *et al*., 1993; Wilson and Cole, 1988). While pyocyanin can lower cAMP and ATP levels of cells (Kantar *et al*., 1994), pyocyanin and 1-hydroxyphenazine also stimulate inflammatory cells to release reactive oxygen species (Jackowski *et al*., 1991). In fact, high concentrations of radicals have been reported to decrease CBF (e.g., Min *et al*., 1999; Burman and Martin, 1986; Kantar *et al*., 1994) and even lower concentrations ($>10\,\mu M$) of hydrogen peroxide reduce CBF (Jackowski *et al*., 1991; Kobayashi *et al*., 1992), possibly by activating PKC (Kobayashi *et al*., 1992). In fact, the finding of decreased CBF upon PKC activation has been consistent in all mammalian cilia examined (Kobayashi *et al*., 1989; Wyatt *et al*., 2000; Wong *et al*., 1998), even though the mechanisms by which PKC inhibits CBF are not fully understood. Whether or not a ciliary membrane phosphorylation target for PKC found in ovine cilia (Salathe *et al*., 1993b) plays a role needs further examination.

Mycoplasma pneumoniae (Biberfeld and Biberfeld, 1970) and viruses especially from the influenza group (Camner *et al*., 1973a) can cause epithelial disruption and mucociliary dysfunction if more than 50 per cent of ciliated cells are destroyed (Battista *et al*., 1972).

Chronic bronchitis

Mucociliary clearance is impaired at least during exacerbations of chronic bronchitis (Svartengren *et al*., 1996; Dirksen *et al*., 1987; Vastag *et al*., 1985; Mossberg *et al*., 1976; Santa Cruz *et al*., 1974) and COPD (Smaldone *et al*., 1993, Camner *et al*., 1973b). Whether or not ciliary dysfunction is involved remains a subject of debate. However, there are detrimental effects on cilia encountered in these diseases.

In chronic bronchitis, airway inflammation with neutrophils and bacterial infections are common. The effects of bacterial products on cilia were discussed above. In addition, neutrophil elastase causes abnormal ciliary function, possibly by disruption of epithelial barriers (Amitani *et al*., 1991; Smallman *et al*., 1984; Tegner *et al*., 1979). Airway infection and inflammation can lead to acquired ciliary disorders including misalignments of the central microtubules between adjacent cilia, compound cilia, and supernumerary microtubules (Afzelius *et al*., 1983), all of which may contribute to mucociliary dysfunction. Finally, airway acidification during COPD and bronchiectasis exacerbations (Kostikas *et al*., 2002) may adversely affect cilia (see above).

Asthma

Mucociliary clearance is also dysfunctional during asthma exacerbations (Ahmed *et al*., 1981). In vitro studies, however, showed that ciliary activity was not depressed upon allergen challenge (Maurer *et al*., 1982b; Wanner *et al*., 1986). In fact, inflammatory mediators usually stimulated ciliary beating (Maurer *et al*., 1982a; Tamaoki *et al*., 1991). There were notable exceptions, however, that caused ciliary dysfunction including platelet activating factor (Seybold *et al*., 1990; Ohashi *et al*., 1994; Ganbo *et al*., 1991), eosinophilic major basic protein (that accumulates in the sputum of asthmatic patients) (Frigas *et al*., 1980, 1981), and leukotriene C_4 in the presence of gamma-glutamyl transpeptidase (Ganbo *et al*., 1996). Furthermore, other serum proteins (including complement C3a and C5), released into the airway lumen during inflammation, decrease CBF (Sanderson and Sleigh, 1981b; Kennedy

et al., 1982). Airway acidification also occurs during exacerbations of asthma (Hunt *et al*., 2000; Ojoo *et al*., 2005) and may affect CBF.

Cystic fibrosis

CBF of cells from patients suffering from cystic fibrosis (CF) has been reported to be normal when measured in vitro (Rutland and Cole, 1981). However, multiple products found in CF airway in vivo can cause ciliary dysfunction including bacterial products and neutrophil elastase (see above). A decrease in the periciliary fluid level, expected at least during exacerbations (Tarran *et al*., 2006), also will impede ciliary function (Matsui *et al*., 1998; Mall *et al*., 2004; Trout *et al*., 2003). Finally, airway acidification during exacerbations (Coakley and Boucher, 2001; Coakley *et al*., 2003) could again lead to ciliary dysfunction.

References

Afzelius BA (1976) A human syndrome caused by immotile cilia. *Science*, **193**, 317–19.

Afzelius BA, Camner P and Mossberg B (1983) Acquired ciliary defects compared to those seen in the immotile-cilia syndrome. *European Journal of Respiratory Diseases*, **64**, 5–10.

Ahmed T, Greenblatt DW, Birch S, Marchette B and Wanner A (1981) Abnormal mucociliary transport in allergic patients with antigen-induced bronchospasm: role of slow reactive substance of anaphylaxis. *American Review of Respiratory Disease*, **124**, 110–14.

Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D, Stockley RA and Cole PJ (1991) Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *American Journal of Respiratory Cell & Molecular Biology*, **4**, 26–32.

Andrade YN, Fernandes J, Vazquez E, Fernandez-Fernandez JM, Arniges M, Sanchez TM, Villalon M and Valverde MA (2005) TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *Journal of Cell Biology*, **168**, 869–74.

Battista SP, Denine EP and Kensler CJ (1972) Restoration of tracheal mucosa and ciliary particle transport activity after mechanical denudation in the chicken. *Journal of Toxicology and Applied Pharmacology*, **22**, 56–69.

Biberfeld C and Biberfeld P (1970) Ultrastructural features of *Mycoplasma pneumoniae*. *Journal of Bacteriology*, **102**, 855–61.

Brokaw CJ and Kamiya R (1987) Bending patterns of *Chlamydomonas flagella*: IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. *Cell Motility and the Cytoskeleton*, **8**, 68–75.

Burman WJ and Martin WJ, II (1986) Oxidant-mediated ciliary dysfunction: possible role in airway disease. *Chest*, **89**, 410–13.

Camner P, Jarstrand C and Philipson K (1973a) Tracheobronchial clearance in patients with influenza. *American Review of Respiratory Disease*, **108**, 69–76.

Camner P, Mossberg B and Philipson K (1973b) Tracheobronchial clearance and chronic obstructive lung disease. *Scand J Respir Dis*, **54**, 272–81.

Chilvers MA, Rutman A and O'Callaghan C (2003) Ciliary beat pattern is associated with specific ultrastructural defects in primary ciliary dyskinesia. *Journal of Allergy and Clinical Immunology*, **112**, 518–24.

Clary-Meinesz C, Mouroux J, Cosson J, Huitorel P and Blaive B (1998) Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. *European Respiratory Journal*, **11**, 330–3.

Coakley RD and Boucher RC (2001) Regulation and functional significance of airway surface liquid pH. *Journal of Physiology*, **2**, 294–300.

Coakley RD, Grubb BR, Paradiso AM, Gatzy JT, Johnson LG, Kreda SM, O'Neal WK and Boucher RC (2003) Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 16083–8.

Di Benedetto G, Manara-Shediac FS and Mehta A (1991) Effect of cyclic AMP on ciliary activity of human respiratory epithelium. *European Respiratory Journal*, **4**, 789–95.

Dirksen H, Hermansen F, Groth S and Molgaard F (1987) Mucociliary clearance in early simple chronic bronchitis. *European Journal of Respiratory Diseases*, **153** Suppl, 145–9.

Dowling RB, Johnson M, Cole PJ and Wilson R (1998) Effect of salmeterol on *Haemophilus influenzae* infection of respiratory mucosa in vitro. *European Respiratory Journal*, **11**, 86–90.

Frigas E, Loegering DA and Gleich GJ (1980) Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Laboratory Investigation*, **42**, 35–43.

Frigas E, Loegering DA, Solley GO, Farrow GM and Gleich GJ (1981) Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. *Mayo Clinic Proceedings*, **56**, 345–53.

Ganbo T, Hisamatsu K, Nakazawa T, Kamijo A and Murakami Y (1991) Platelet activating factor (PAF) effects on ciliary activity of human paranasal sinus mucosa in vitro. *Rhinology*, **29**, 231–7.

Ganbo T, Hisamatsu K and Inoue H (1996) Leukotriene C-4 inhibits ciliary activity in the presence of gamma-glutamyl transpeptidase. *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **15**, L216–L224.

Geary CA, Davis CW, Paradiso AM and Boucher RC (1995) Role of CNP in human airways: cGMP-mediated stimulation of ciliary beat frequency. *American Journal of Physiology*, **268**, L1021–8.

Gheber L and Priel Z (1989) Synchronization between beating cilia. *Biophysical Journal*, **55**, 183–91.

Gheber L and Priel Z (1994) Metachronal activity of cultured mucociliary epithelium under normal and stimulated conditions. *Cell Motility and the Cytoskeleton*, **28**, 333–345.

Gheber L, Korngreen A and Priel Z (1998) Effect of viscosity on metachrony in mucus propelling cilia. *Cell Motility and the Cytoskeleton*, **39**, 9–20.

Hastie AT, Marchese-Ragona SP, Johnson KA and Wall JS (1988) Structure and mass of mammalian respiratory ciliary outer arm 19S dynein. *Cell Motility and the Cytoskeleton*, **11**, 157–66.

Hingley ST, Hastie AT, Kueppers F, Higgins ML, Weinbaum G and Shryock T (1986) Effect of ciliostatic factors from *Pseudomonas aeruginosa* on rabbit respiratory cilia. *Infection and Immunity*, **51**, 254–62.

Hirokawa N, Tanaka Y, Okada Y and Takeda S (2006) Nodal flow and the generation of left–right asymmetry. *Cell*, **125**, 33–45.

Horvath G, Schmid N, Fragoso MA, Schmid A, Conner GE, Salathe M and Wanner A (2006) Epithelial organic cation transporters ensure pH dependent drug absorption in the airway. *American Journal of Respiratory Cell and Molecular Biology*, **36**, 53–60.

Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TA and Gaston B (2000) Endogenous airway acidification. Implications for asthma pathophysiology. *American Journal of Respiratory and Critical Care Medicine*, **161**, 694–9.

Jackowski JT, Szepfalusi ZS, Wanner DA, Seybold ZV, Sielczak MW, Lauredo IT, Adams T, Abraham WM and Wanner A (1991) Effects of *P. aeruginosa*-derived bacterial products on tracheal ciliary function: role of O_2 radicals. *American Journal of Physiology*, **260**, L61–7.

Johnson NT, Villalon M, Royce FH, Hard R and Verdugo P (1991) Autoregulation of beat frequency in respiratory ciliated cells. Demonstration by viscous loading. *American Review of Respiratory Disease*, **144**, 1091–4.

Kamiya R (2002) Functional diversity of axonemal dyneins as studied in *Chlamydomonas* mutants. *International Review of Cytology*, **219**, 115–55.

Kantar A, Oggiano N, Giorgi PL, Braga PC and Fiorini R (1994) Polymorphonuclear leukocyte-generated oxygen metabolites decrease beat frequency of human respiratory cilia. *Lung*, **172**, 215–22.

Kanthakumar K, Taylor G, Tsang KW, Cundell DR, Rutman A, Smith S, Jeffery PK, Cole PJ and Wilson R (1993) Mechanisms of action of *Pseudomonas aeruginosa* pyocyanin on human ciliary beat in vitro. *Infection and Immunity*, **61**, 2848–53.

Kennedy JR, Lin KD and Duckett KE (1982) Serum complement (C3a and C5)-induced inhibition of rabbit tracheal cilia. *Progress in Clinical and Biological Research*, **80**, 71–5.

Keskes L, Giroux-Widemann V, Serres C, Pignot-Paintrand I, Jouannet P and Feneux D (1998) The reactivation of demembranated human spermatozoa lacking outer dynein arms is independent of pH. *Molecular Reproduction and Development*, **49**, 416–25.

Kienast K, Riechelmann H, Knorst M, Schlegel J, Mullerquernheim J, Schellenberg J and Ferlinz R (1994) An experimental model for the exposure of human ciliated cells to sulfur dioxide at different concentrations. *Clinical Investigator*, **72**, 215–19.

Kobayashi K, Tamaoki J, Sakai N, Chiyotani A and Takizawa T (1989) Inhibition of ciliary activity by phorbol esters in rabbit tracheal cells. *Lung*, **167**, 277–84.

Kobayashi K, Salathe M, Pratt MM, Cartagena NJ, Soloni F, Seybold ZV and Wanner A (1992) Mechanism of hydrogen peroxide-induced inhibition of sheep airway cilia. *American Journal of Respiratory Cell and Molecular Biology*, **6**, 667–73.

Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P and Loukides S (2002) pH in expired breath condensate of patients with inflammatory airway diseases. *American Journal of Respiratory and Critical Care Medicine*, **165**, 1364–70.

Lansley AB and Sanderson MJ (1999) Regulation of airway ciliary activity by Ca^{2+}: simultaneous measurement of beat frequency and intracellular Ca^{2+}. *Biophysical Journal*, **77**, 629–38.

Lieb T, Wijkstrom Frei C, Frohock JI, Bookman RJ and Salathe M (2002) Prolonged increase in ciliary beat frequency after short-term purinergic stimulation in human airway epithelial cells. *Journal of Physiology*, **538**, 633–646.

Ma W, Silberberg SD and Priel Z (2002) Distinct axonemal processes underlie spontaneous and stimulated airway ciliary activity. *Journal of General Physiology*, **120**, 875–85.

Mall M, Grubb BR, Harkema JR, O'Neal WK and Boucher RC (2004) Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. *Nature Medicine*, **10**, 487–93.

Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW and Boucher RC (1998) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell*, **95**, 1005–15.

Maurer DR, Schor J, Sielczak M, Wanner A and Abraham WM (1982a) Ciliary motility in airway anaphylaxis. *Progress in Clinical and Biological Research*, **80**, 67–70.

Maurer DR, Sielczak M, Oliver W, Jr, Abraham WM and Wanner A (1982b) Role of ciliary motility in allergic mucociliary dysfunction. *Journal of Applied Physiology*, **52**, 1018–23.

McEwen BF, Radermacher M, Rieder CL and Frank J (1986) Tomographic three-dimensional reconstruction of cilia ultrastructure from thick sections. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 9040–4.

McGrath J, Somlo S, Makova S, Tian X and Brueckner M (2003) Two populations of node monocilia initiate left–right asymmetry in the mouse. *Cell*, **114**, 61–73.

Min YG, Ohyama M, Lee KS, Rhee CS, Oh SH, Sung MW, Yun JB and Jung IH (1999) Effects of free radicals on ciliary movement in the human nasal epithelial cells. *Auris Nasus Larynx*, **26**, 159–63.

Mitchison H, Salathe M, Leigh M and Carson J (2006) Primary ciliary dyskinesia. In: *Encyclopedia of Respiratory Medicine*. Laurent G and Shapiro S (eds), New York, Elsevier, pp. 485–489.

Moller W, Haussinger K, Ziegler-Heitbrock L and Heyder J (2006) Mucociliary and long-term particle clearance in airways of patients with immotile cilia. *Respiratory Research*, **7**, 10.

Morse DM, Smullen JL and Davis CW (2001) Differential effects of UTP, ATP, and adenosine on ciliary activity of human nasal epithelial cells. *American Journal of Physiology*, **280**, C1485–97.

Mossberg B, Strandberg K, Philipson K and Camner P (1976) Tracheobronchial clearance and beta-adrenoceptor stimulation in patients with chronic bronchitis. *Scandinavian Journal of Respiratory Diseases*, **57**, 281–9.

Nicastro D, Schwartz C, Pierson J, Gaudette R, Porter ME and McIntosh JR (2006) The molecular architecture of axonemes revealed by cryoelectron tomography. *Science*, **313**, 944–8.

Nogales E, Whittaker M, Milligan RA and Downing KH (1999) High-resolution model of the microtubule. *Cell*, **96**, 79–88.

Nonaka S, Tanaka Y, Okada Y, Takeda S, Harada A, Kanai Y, Kido M and Hirokawa N (1998) Randomization of left–right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell*, **95**, 829–37.

Noone PG, Leigh MW, Sannuti A, Minnix SL, Carson JL, Hazucha M, Zariwala MA and Knowles MR (2004) Primary ciliary dyskinesia: diagnostic and phenotypic features. *American Journal of Respiratory and Critical Care Medicine*, **169**, 459–67.

Ohashi Y, Ohno Y, Sugiura Y, Okamoto H, Tanaka A, Kakinoki Y, Sakamoto H, Hayashi M and Nakai Y (1994) Presence of bronchoalveolar lavage fluid necessary for platelet activating factor-induced ciliary depression. *International Archives of Allergy and Immunology*, **104**, 302–306.

Ojoo JC, Mulrennan SA, Kastelik JA, Morice AH and Redington AE (2005) Exhaled breath condensate pH and exhaled nitric oxide in allergic asthma and in cystic fibrosis. *Thorax*, **60**, 22–6.

Ostrowski LE, Blackburn K, Radde KM, Moyer MB, Schlatzer DM, Moseley A and Boucher RC (2002) A proteomic analysis of human cilia: identification of novel components. *Molecular and Cellular Proteomics*, **1**, 451–65.

Padayatti PS, Pattanaik P, Ma X and van den Akker F (2004) Structural insights into the regulation and the activation mechanism of mammalian guanylyl cyclases. *Pharmacology and Therapeutics*, **104**, 83–99.

Pazour GJ, Agrin N, Leszyk J and Witman GB (2005) Proteomic analysis of a eukaryotic cilium. *Journal of Cell Biology*, **170**, 103–13.

Piperno G, Huang B, Ramanis Z and Luck DJ (1981) Radial spokes of *Chlamydomonas flagella*: polypeptide composition and phosphorylation of stalk components. *Journal of Cell Biology*, **88**, 73–9.

Rosenbaum JL and Witman GB (2002) Intraflagellar transport. *Nature Reviews. Molecular Cell Biology*, **3**, 813–25.

Rutland J and Cole PJ (1981) Nasal mucociliary clearance and ciliary beat frequency in cystic fibrosis compared with sinusitis and bronchiectasis. *Thorax*, **36**, 654–8.

Salathe M (2002) Effects of beta-agonists on airway epithelial cells. *Journal of Allergy and Clinical Immunology*, **110**, S275–81.

Salathe M and Bookman RJ (1999) Mode of Ca^{2+} action on ciliary beat frequency in single ovine airway epithelial cells. *Journal of Physiology*, **520**, 851–65.

Salathe M, Pratt MM and Wanner A (1993a) Cyclic AMP-dependent phosphorylation of a 26 kDa axonemal protein in ovine cilia isolated from small tissue pieces. *American Journal of Respiratory Cell and Molecular Biology*, **9**, 306–14.

Salathe M, Pratt MM and Wanner A (1993b) Protein kinase C-dependent phosphorylation of a ciliary membrane protein and inhibition of ciliary beating. *Journal of Cell Science*, **106**, 1211–20.

Sanderson MJ and Dirksen ER (1985) A versatile and quantitative computer-assisted photoelectronic technique used for the analysis of ciliary beat cycles. *Cell Motility*, **5**, 267–92.

Sanderson MJ and Dirksen ER (1989) Mechanosensitive and beta-adrenergic control of the ciliary beat frequency of mammalian respiratory tract cells in culture. *American Review of Respiratory Disease*, **139**, 432–40.

Sanderson MJ and Sleigh MA (1981a) Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. *Journal of Cell Science*, **47**, 331–47.

Sanderson MJ and Sleigh MA (1981b) Serum proteins agglutinate cilia and modify ciliary coordination. *Pediatric Research*, **15**, 219–28.

Santa Cruz R, Landa J, Hirsch J and Sackner MA (1974) Tracheal mucous velocity in normal man and patients with obstructive lung disease: effects of terbutaline. *American Review of Respiratory Disease*, **109**, 458–63.

Satir P and Christensen ST (2006) Overview of structure and function of mammalian cilia. *Annual Review of Physiology*, **69**, 377–400.

Satir P and Sleigh MA (1990) The physiology of cilia and mucociliary interactions. *Annual Review of Physiology*, **52**, 137–55.

Schmid A, Sutto Z, Conner GE, Fregien N and Salathe M (2005) Ciliary beat frequency regulation by soluble adenylyl cyclase in normal and CF airway epithelia. *Pediatric Pulmonology*, **28** Suppl, 226.

Scholey JM and Anderson KV (2006) Intraflagellar transport and cilium-based signaling. *Cell*, **125**, 439–42.

Seybold ZV, Mariassy AT, Stroh D, Kim CS, Gazeroglu H and Wanner A (1990) Mucociliary interaction in vitro: effects of physiological and inflammatory stimuli. *Journal of Applied Physiology*, **68**, 1421–6.

Siewert A (1904) Über einen Fall von Bronchiektasie bei einem Patienten mit situs inversus viscerum. *Berliner Klinische Wochenschrift*, **41**, 139–141.

Smaldone GC, Foster WM, O'Riordan TG, Messina MS, Perry RJ and Langenback EG (1993) Regional impairment of mucociliary clearance in chronic obstructive pulmonary disease. *Chest*, **103**, 1390–6.

Smallman LA, Hill SL and Stockley RA (1984) Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect. *Thorax*, **39**, 663–7.

Stephens R (1970) Isolation of nexin – the linkage protein responsible for the maintenance of the nine-fold configuration of flagellar axonemes. *The Biological Bulletin*, **139**, 438.

Supp DM, Witte DP, Potter SS and Brueckner M (1997) Mutation of an axonemal dynein affects left-right asymmetry in inversus viscerum mice. *Nature*, **389**, 963–6.

Sutto Z, Conner GE and Salathe M (2004) Regulation of human airway ciliary beat frequency by intracellular pH. *Journal of Physiology*, **560**, 519–532.

Svartengren K, Ericsson CH, Svartengren M, Mossberg B, Philipson K and Camner P (1996) Deposition and clearance in large and small airways in chronic bronchitis. *Experimental Lung Research*, **22**, 555–576.

Tamaoki J, Sakai N, Kobayashi K, Kanemura T and Takizawa T (1991) Stimulation of airway ciliary motility by immunologically activated canine pulmonary macrophages: role of leukotrienes. *Acta Physiologica Scandinavica*, **141**, 415–20.

Tarran R, Button B and Boucher RC (2006) Regulation of normal and cystic fibrosis airway surface liquid volume by phasic shear stress. *Annual Review of Physiology*, **68**, 543–61.

Taylor BL and Zhulin IB (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiology and Molecular Biology Reviews*, **63**, 479–506.

Tegner H, Ohlsson K, Toremalm NG and von Mecklenburg C (1979) Effect of human leukocyte enzymes on tracheal mucosa and its mucociliary activity. *Rhinology*, **17**, 199–206.

Teilmann SC and Christensen ST (2005) Localization of the angiopoietin receptors Tie-1 and Tie-2 on the primary cilia in the female reproductive organs. *Cell Biology International*, **29**, 340–6.

Teilmann SC, Byskov AG, Pedersen PA, Wheatley DN, Pazour GJ and Christensen ST (2005) Localization of transient receptor potential ion channels in primary and motile cilia of the female murine reproductive organs. *Molecular Reproduction and Development*, **71**, 444–52.

Trout L, Townsley MI, Bowden AL and Ballard ST (2003) Disruptive effects of anion secretion inhibitors on airway mucus morphology in isolated perfused pig lung. *Journal of Physiology*, **549**, 845–53.

Vastag E, Matthys H, Kohler D, Gronbeck L and Daikeler G (1985) Mucociliary clearance and airways obstruction in smokers, ex-smokers and normal subjects who never smoked. *European Journal of Respiratory Diseases*, **139** Suppl, 93–100.

Wais-Steider J and Satir P (1979) Effect of vanadate on gill cilia: switching mechanism in ciliary beat. *Journal of Supramolecular Structure*, **11**, 339–47.

Wanner A, Sielczak M, Mella JF and Abraham WM (1986) Ciliary responsiveness in allergic and non-allergic airways. *Journal of Applied Physiology*, **60**, 1967–71.

Warner FD (1976) Ciliary inter-microtubule bridges. *Journal of Cell Science*, **20**, 101–14.

Wilson R and Cole PJ (1988) The effect of bacterial products on ciliary function. *American Review of Respiratory Disease*, **138**, 49–53.

Wilson R, Roberts D and Cole P (1985) Effect of bacterial products on human ciliary function in vitro. *Thorax*, **40**, 125–31.

Wilson R, Pitt T, Taylor G, Mc Dermot J, Sykes D, Roberts D and Cole P (1987) Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. *Journal of Clinical Investigation*, **79**, 221–9.

Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW and Cole PJ (1988) Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infection and Immunology*, **56**, 2515–17.

Wong LB, Miller IF and Yeates DB (1993) Nature of the mammalian ciliary metachronal wave. *Journal of Applied Physiology*, **75**, 458–67.

Wong LB, Park CL and Yeates DB (1998) Neuropeptide Y inhibits ciliary beat frequency in human ciliated cells via nPKC, independently of PKA. *American Journal of Physiology*, **275**, C440–8.

Wyatt TA, Spurzem JR, May K and Sisson JH (1998) Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells. *American Journal of Physiology*, **275**, L827–35.

Wyatt TA, Schmidt SC, Rennard SI, Tuma DJ and Sisson JH (2000) Acetaldehyde-stimulated PKC activity in airway epithelial cells treated with smoke extract from normal and smokeless cigarettes. *Proceedings of the Society for Experimental Biology and Medicine*, **225**, 91–7.

Wyatt TA, Forget MA, Adams JM and Sisson JH (2005) Both cAMP and cGMP are required for maximal ciliary beat stimulation in a cell-free model of bovine ciliary axonemes. *American Journal of Physiology – Lung Cell and Molecular Physiology*, **288**, L546–51.

Yang P, Diener DR, Rosenbaum JL and Sale WS (2001) Localization of calmodulin and dynein light chain LC8 in flagellar radial spokes. *Journal of Cell Biology*, **153**, 1315–26.

Yang P, Diener DR, Yang C, Kohno T, Pazour GJ, Dienes JM, Agrin NS, King SM, Sale WS, Kamiya R, Rosenbaum JL and Witman GB (2006) Radial spoke proteins of *Chlamydomonas* flagella. *Journal of Cell Science*, **119**, 1165–74.

Zariwala MA, Knowles MR and Omran H (2006) Genetic defects in ciliary structure and function. *Annual Review of Physiology*, **69**, 323–50.

Zhang L and Sanderson MJ (2003a) Oscillations in ciliary beat frequency and intracellular calcium concentration in rabbit tracheal epithelial cells induced by ATP. *Journal of Physiology*, **546**, 733–49.

Zhang L and Sanderson MJ (2003b) The role of cGMP in the regulation of rabbit airway ciliary beat frequency. *Journal of Physiology*, **551**, 765–776.

7

Composition and Function of Airway Mucus

Duncan F. Rogers
National Heart & Lung Institute, Imperial College London, UK

Inhalation of ~500 L of air an hour bombards the airway epithelium with up to 600 million particles a day (Seaton *et al*., 1995). Cigarette-smoking more than doubles that amount (Hollander and Stober, 1986; Lippmann *et al*., 1980). As a result, the airway epithelium has developed ways to combat this onslaught of soot, dust, microbes and allergens. First-line defence against an inhaled insult impinging on, and causing damage to, the epithelium is the production of mucus. This mucus is a viscoelastic gel that forms a thin film that overlies the internal surface of the airway (Figure 7.1). It is an important homeostatic defence mechanism with a variety of functions (Table 7.1) that have evolved to reduce potential epithelial damage by inhaled irritants. Under normal circumstances airway mucus protects the epithelial lining by entrapping foreign debris, bacteria and viruses and clearing them from the airway by ciliary movement (Rose and Voynow, 2006). In contrast, in clinical conditions associated with airway mucus hypersecretion, for example asthma (Del Donno *et al*., 2000), chronic obstructive pulmonary disease (COPD) (Houtmeyers *et al*., 1999; Maestrelli *et al*., 2001) and cystic fibrosis (CF) (Robinson and Bye, 2002), the mucus shifts from a protective role to one that contributes to respiratory disease. Excessive production of airway mucus, termed mucus hypersecretion, and changes in the biophysical properties of the mucus, can lead to decreased mucociliary clearance and accumulation of mucus in the lungs (Figure 7.2), leading to difficulty in breathing, increased morbidity and, in severe cases, increased mortality. The latter aspects are covered in the present chapter and are introduced in the following sections.

7.1 Airway 'mucus'

Airway mucus is a complex dilute aqueous solution of lipids, glycoconjugates and proteins. It comprises salts, enzymes and anti-enzymes, oxidants and antioxidants, exogenous bacterial

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

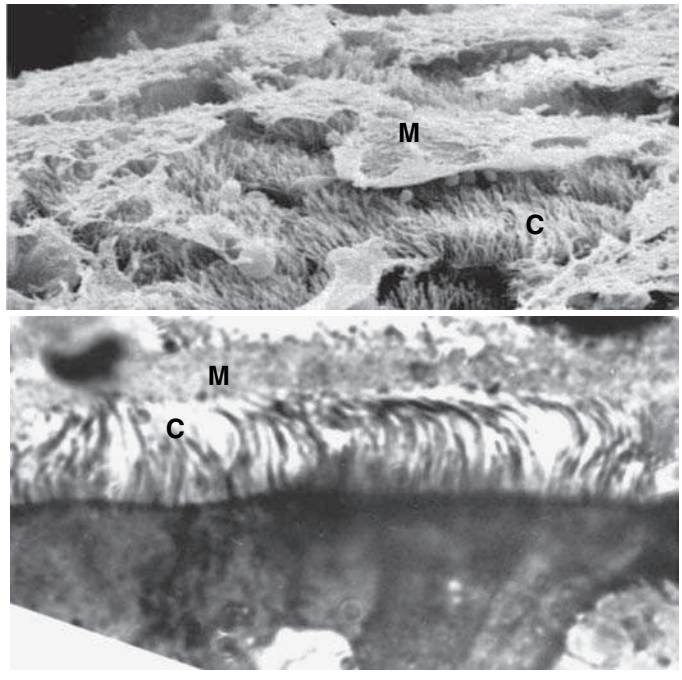


Figure 7.1 Visualization of airway luminal mucus and cilia. Upper plate: Scanning electron micrograph of human bronchus showing mucus 'flakes' or 'rafts' (M) resting on top of cilia (C) (courtesy of P. K. Jeffery). Lower plate: Mucociliary clearance in bovine trachea. Mucus (M) sits on top of the cilia (C), which are seen bent at different stages of the beat cycle (courtesy of K. Pritchard)

Table 7.1 Functions of airway mucus

Physical barrier to inhaled airborne organisms, particles and other irritants, as well as to aspirated foods and liquids
Entrapment of organisms, particles and irritants
Formation of the vehicle on which irritants are transported by mucociliary activity for clearance from the airway
Provision of a waterproof layer over the epithelium to limit dehydration
Humidification of inspired air
Insulation
pH-buffering capacity
Lubrication
Neutralization of toxic gases
Selective macromolecular sieve
Source of antibacterial and other protective enzymes, and provision of extracellular surface for their activity
Source of immunoglobulins, and provision of extracellular surface for their activity

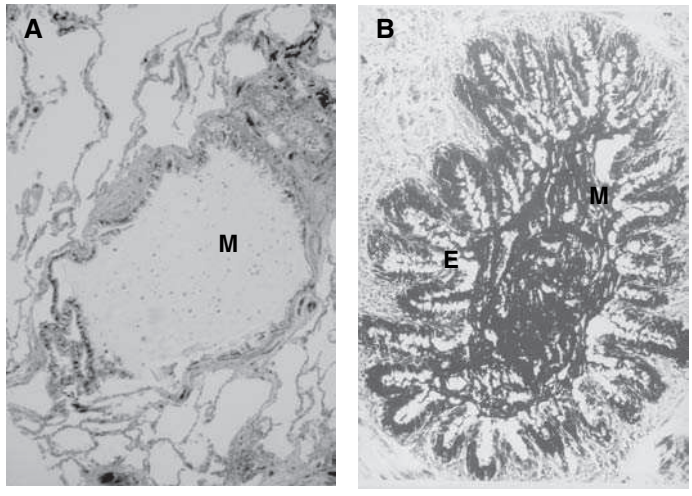


Figure 7.2 Mucus obstruction of the airway in COPD and asthma. (A) Mucus obstruction (M) in an intrapulmonary airway of a cigarette smoker. (B) Fatal interaction between bronchoconstriction and luminal mucus. Intrapulmonary airway of a patient who died of an acute severe asthma attack showing airway epithelium (E) thrown into folds by smooth muscle contraction, and occlusion of remaining lumen by mucus (M)

products, endogenous antibacterial secretions, cell-derived mediators and proteins, plasma-derived mediators and proteins, and cell debris such as DNA. Airway mucus is considered to form a liquid bi-layer whereby an upper gel layer floats above a lower, more watery sol, or periciliary liquid, layer (Knowles and Boucher, 2002). The functions of the sol layer are debated, but are presumed to include 'lubrication' of the beating cilia. The gel layer traps particles and is moved on the tips of the beating cilia. The inhaled particles are trapped in the sticky gel layer and are removed from the airway by mucociliary clearance. When the mucus reaches the throat, it is either swallowed and delivered to the gastrointestinal tract for degradation or, if excessive, as in respiratory disease, it is coughed out (Rose and Voynow, 2006).

Respiratory tract mucus requires the correct combination of viscosity and elasticity for optimal efficiency of ciliary interaction. Viscosity is a liquid-like characteristic and is the resistance to flow and the capacity to absorb energy when moving. Elasticity is a solid-like property and is the capacity to store the energy used to move or deform it. Viscoelasticity confers a number of properties to the mucus that allow effective interaction with cilia. These properties have been variously described in terms of spinnability, adhesiveness and wettability (Houtmeyers *et al*., 1999). An important characteristic of mucus is that it is non-Newtonian: its viscosity decreases as the applied force increases (Sleigh *et al*., 1988). Consequently, the ratio of stress to rate of strain is nonlinear, with the result that the more forcefully the cilia beat, the more easily the mucus moves. Viscoelasticity is conferred on the mucus primarily by high molecular weight mucous glycoproteins, termed mucins (Figure 7.3).

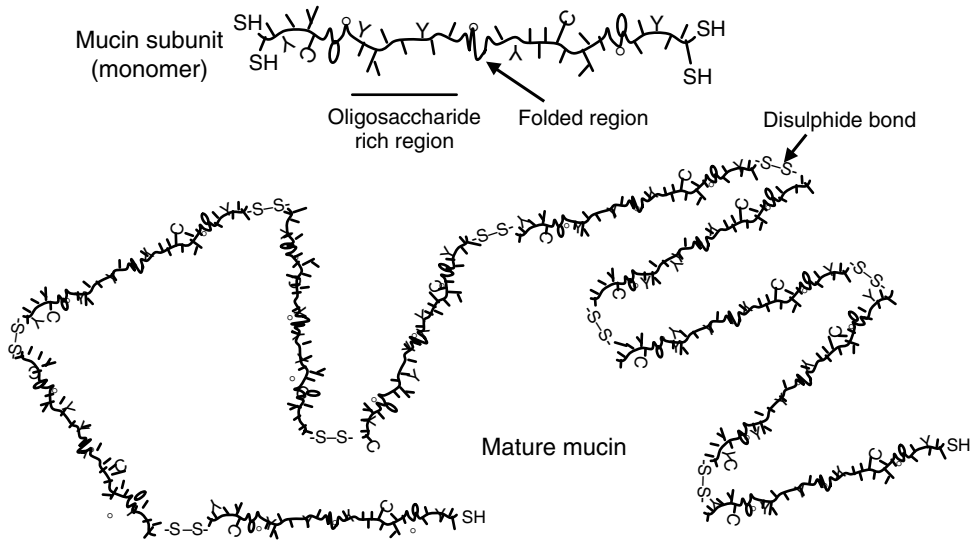


Figure 7.3 Schematic representation of a gel-forming mucin molecule. The mucin subunit (~500 nm in length) comprises an amino acid backbone with highly glycosylated (linear) domains and folded regions, stabilized via disulphide bonds, with little or no glycosylation. Glycosylation is via O-linkages and is highly diverse. In secretions, the subunits are joined end-to-end by disulphide bonds (S—S) to form long, thread-like mature mucin molecules

7.2 Respiratory tract mucins

In health, mucins comprise up to 2 per cent by weight of the airway mucus (Davies *et al*., 2002). In the airway, mucins are produced by goblet cells in the epithelium (Rogers, 2003) (Figure 7.4) and sero-mucous glands in the submucosa (Finkbeiner, 1999). Although the emphasis of the present book is the pulmonary epithelium, submucosal glands will be mentioned herein where relevant as a comparison with goblet cells.

Mucins are long, thread-like, complex glycoconjugates (Figure 7.3). They consist of a linear peptide backbone (termed apomucin) encoded by specific mucin (MUC) genes (see below), to which hundreds of carbohydrate side-chains are O-linked, but also with additional N-linked glycans. The glycosylation pattern is complex and extremely diverse (Hanisch, 2001), and is associated with complementary motifs on bacterial cell walls, thereby facilitating broad-spectrum bacterial attachment and subsequent clearance (Dell and Morris, 2001; Moniaux *et al*., 2001). Within the main protein core are variable numbers of tandemly-repeated serine- and/or threonine-rich regions which are unique in size and sequence for each mucin (Rose and Voynow, 2006), and represent sites for mucin glycosylation. These complex glycoproteins are polydisperse, linear polymers that can be fragmented by reduction to give monomers termed 'reduced subunits' (Sheehan *et al*., 1991, Thornton *et al*., 1990, 1991, 1994). There are at least two structurally and functionally distinct classes of mucin, namely the membrane-associated mucins (Table 7.2) and the secreted (gel-forming or non gel-forming) mucins (Tables 7.3 and 7.4). Membrane-tethered mucins, which have a hydrophobic domain that anchors the mucin in the plasma membrane, contribute to the composition of

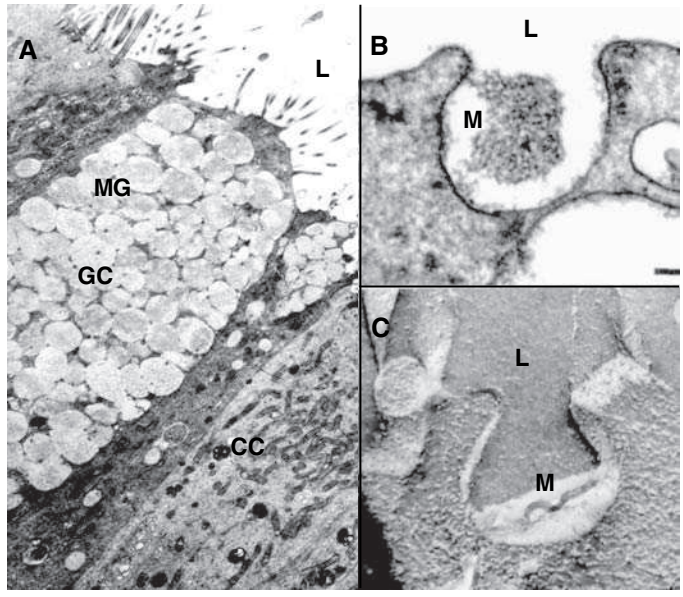


Figure 7.4 Airway goblet cells. (A) Goblet cell (GC) and ciliated cell (CC) in human bronchial epithelium. MG = mucin granule, L = lumen. Transmission electron micrograph after gluteraldehyde fixation and post-fixation in osmium tetroxide. (B and C) Exocytosis of mucin (M) by guinea-pig tracheal goblet cell, visualized after tannic acid incubation, demonstrating 'omega' profile formed by fusion of intracellular granule and apical membrane: ultrathin section (B) and freeze-fracture replication (C). L = lumen

Table 7.2 Human MUC genes producing membrane-associated mucins

Gene	Tissue distribution
MUC 1	Lung, cornea, salivary glands, oesophagus, stomach, pancreas, large intestine, breast, prostate, ovary, kidney, uterus, cervix, dendritic cells
MUC 3A	Thymus, small intestine, colon, kidney
MUC 3B	Small intestine, colon
MUC 4	Lung, cornea, salivary glands, oesophagus, small intestine, kidney, endocervix
MUC 11	Lung, middle ear, thymus, small intestine, pancreas, colon, liver, kidney, uterus, prostate
MUC 12	Middle ear, pancreas, colon, uterus, prostate
MUC 13	Lung, conjunctiva, stomach, small intestine, colon, kidney
MUC 15	Conjunctiva, tonsils, thymus, lymph node, breast, small intestine, colon, liver, spleen, prostate, testis, ovary, leukocytes, bone marrow
MUC 16	Conjunctiva, ovary
MUC 17	Intestinal cells, conjunctival epithelium
MUC 18	Prostate
MUC 20	Lung, liver, kidney, colon, placenta, prostate

Table 7.3 Human MUC genes producing secreted, cysteine-rich (gel-forming) mucins

Gene	Tissue distribution
MUC 2	Lung, conjunctiva, middle ear, stomach, small intestine, colon, nasopharynx, prostate
MUC 5AC	Lung, conjunctiva, middle ear, stomach, gall bladder, nasopharynx
MUC 5B	Lung, middle ear, sublingual gland, laryngeal submucosal, glands, oesophageal glands, stomach, duodenum, gall bladder, nasopharynx
MUC 6	Stomach, duodenum, gall bladder, pancreas, kidney
MUC 19	Lung, salivary gland, kidney, liver, colon, placenta, prostate

Table 7.4 Human MUC genes producing secreted, cysteine-poor mucins

Gene	Tissue distribution
MUC 7	Lung, lachrymal glands, salivary glands, nose
MUC 8	Oviduct
MUC 9	Submandibular glands

the cell surface (Rose and Voynow, 2006). Secretory mucins are stored intracellularly in secretory granules and are released at the apical surface of the cell in response to stimuli. It would appear that mucus production is such a fundamental homeostatic process that virtually all acute interventions examined trigger airway mucin secretion (Table 7.5). In addition, many of these same mediators when administered more chronically not only induce mucin secretion but also upregulate mucin gene expression, with concomitant increases in mucin synthesis: the latter is associated with goblet cell hyperplasia (Table 7.5).

Table 7.5 Inducers of airway mucus secretion, goblet cell hyperplasia and mucin (MUC) synthesis/gene expression

Stimulation	Secretion	Hyperplasia	MUC
Cytokines			
Interleukin (IL)-1β	+	NP	NP
IL-6	+	NP	Yes
IL-9	NP	NP	Yes
IL-13 (IL-4)	+	Yes	Yes
TNFα	++	Yes[a]	Yes[a]
Gases			
Irritant gases (e.g. cigarette smoke)	++	Yes	Yes
Nitric oxide	−ve/+	NP	NP
Reactive oxygen species	0/+	NP	NP
Inflammatory mediators			
Bradykinin	+	NP	NP
Cysteinyl leukotrienes	++	NP	NP
Endothelin	0/+	NP	NP
Histamine	+	NP	NP

PAF	+	Yes[a]	Yes[a]
Prostaglandins	0/+	NP	NP
Proteinases	+++	Yes	NP
Purine nucleotides	++	NP	NP
Neural pathways			
Cholinergic nerves	++	NP	NP
Cholinoceptor agonists	++	Yes	NP
Nicotine	++	Yes	NP
Tachykininergic nerves	+	NP	NP
Substance P	++	NP	NP
Neurokinin A	+	NP	NP
Miscellaneous			
EGF (+ TNFα)	NP	Yes	Yes
Sensitization followed by challenge	+	Yes	Yes

+++ highly potent, ++ marked effect, + lesser effect, 0 minimal effect. NP, effect not published.

[a] Effect only observed with PAF (platelet activating factor) and tumour necrosis factor-α (TNFα) in combination.

EGF, epidermal growth factor; IL, interleukin.

7.3 Mucin genes and gene products

Twenty human mucin (MUC) genes have so far been identified (Tables 7.2–7.4). Of these, only nine, namely MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, MUC8, MUC11 and MUC13, are expressed in the human respiratory tract (Rose and Voynow, 2006). Of these, only MUC2, MUC5AC and MUC5B, the classic gel-forming mucins, are found in airway secretions. However, only MUC5AC and MUC5B glycoproteins, localized adjacent to each other on chromosome 11p15.5, are considered the major gel-forming mucins in both normal respiratory tract secretions as well as in airway secretions from patients with respiratory diseases (Hovenberg *et al*., 1996b, 1996a; Sheehan *et al*., 1999; Thornton *et al*., 1996, 1997; Wickstrom *et al*., 1998). Small amounts of MUC2 may, however, be found in secretions from 'irritated' airway (see below).

In general, the MUC gene products are poorly characterized biochemically and biophysically (Davies *et al*., 2002). The predicted sequences of the MUC1, 3, 4, 8, 11 and 13 gene products suggest they are membrane-bound, with an extracellular mucin domain and a hydrophobic membrane-spanning domain (Table 7.2). In contrast, MUC2, 5AC, 5B, 6 and 7 gene products are secreted mucins (Table 7.3). The technology for studying the contribution to physiology and pathophysiology of the individual MUC gene products lags well behind that of investigation of gene expression (Rose and Voynow, 2006). MUC1, 2, and 8 genes are expressed in both the epithelium and submucosal glands, whereas MUC4, 5AC and 13 are expressed primarily in the epithelium. In contrast, MUC5B and MUC7 genes are expressed primarily in the glands. Use of currently available antibodies confirms that the MUC5AC gene product is a goblet cell mucin, whilst MUC5B predominates in the glands, albeit that some MUC5AC and MUC7 is also usually present (Davies *et al*., 2002).

Interestingly, MUC4 mucin localizes to the ciliated cells. The mucin content of secretions from patients with hypersecretory respiratory diseases may differ from normal (see below).

7.4 MUC5AC

MUC5AC mucin, initially isolated as a tracheobronchial mucin (Guyonnet *et al*., 1995), is found in airway secretions pooled from healthy individuals (Hovenberg *et al*., 1996b; Thornton *et al*., 1996). Increased levels of MUC5AC protein have also been shown to be present in the airway of patients with asthma (Ordonez *et al*., 2001), which suggests that this mucin may contribute to the pathophysiology of asthma. MUC5AC is the main mucin produced by the goblet cells in the tracheobronchial surface epithelium. However, MUC5AC can be found highly expressed not only in human bronchial epithelium, but also in bronchial submucosal glands, nasal mucosa, gastric epithelium, endocervix epithelium and submucosal glands. This mucin has been found to be highly oligomerized, which makes it an ideal gel-forming molecule. The expression of many genes, such as MUC5AC, in airway epithelial cells is regulated by various neurohumoral factors and inflammatory mediators (Table 7.5).

7.5 MUC5B

MUC5B mucins are also a major component of tenacious mucus plug from the lungs of a patient who died in *status asthmaticus* (Sheehan *et al*., 1995; Thornton *et al*., 1997) and in sputum from patients with chronic bronchitis (Wickstrom *et al*., 1998), which suggests that MUC5B is a major component of lung mucus from patients with obstructive lung diseases (Rose *et al*., 2001). MUC5B mucin exists as differently charged glycoforms (termed the low-charge and high-charge glycoforms) and is secreted primarily by the mucous cells in the bronchial submucosal glands (Desseyn *et al*., 1998; Hovenberg *et al*., 1996a; Sharma *et al*., 1998; Thornton *et al*., 1997). However, it has been shown that MUC5B mucins are also synthesized by goblet cells (Wickstrom *et al*., 1998), and are expressed in the tracheal and bronchial glands, salivary glands, endocervix, gall bladder and pancreas. MUC5B is unique in that it does not appear to be polymorphic.

From the above, it appears that, in healthy individuals, MUC5B is mainly expressed in the airway submucosal glands, which are restricted to the more proximal, cartilaginous airway. In contrast, MUC5AC expression is generally restricted to goblet cells in the upper and lower respiratory tracts (Audie *et al*., 1993; Reid *et al*., 1997). Thus, the composition of normal mucus can be altered depending on the relative contribution to the secretions of these different cellular sources (Kirkham *et al*., 2002). In respiratory diseases associated with airway mucus hypersecretion, such as asthma, COPD and CF, further changes in the composition of the mucus, and in the mucus secretory phenotype in general, are observed, as discussed below.

7.6 Airway mucus hypersecretory phenotype in COPD

COPD comprises three overlapping conditions, namely chronic bronchitis (airway mucus hypersecretion), chronic bronchiolitis (small airway disease) and emphysema (airspace

enlargement due to alveolar destruction) (Global Initiative for Chronic Obstructive Lung Disease, 2006). The following discussion considers the 'bronchitic' component of COPD. The airway of patients with COPD contain excessive amounts of mucus (Reid, 1954), which is markedly increased above that in control subjects (Aikawa *et al*., 1989; Steiger *et al*., 1994). The excessive luminal mucus is associated with increased amounts of mucus secreting tissue. Goblet cell hyperplasia is a cardinal feature of chronic bronchitis (Reid, 1954), with increased numbers of goblet cells in the airway of cigarette smokers either with chronic bronchitis and chronic airflow limitation (Saetta *et al*., 2000) or with or without productive cough (Mullen *et al*., 1987). Submucosal gland hypertrophy also characterizes chronic bronchitis (Aikawa *et al*., 1989; Reid, 1954, 1960; Restrepo and Heard, 1963), and the amount of gland correlates with the amount of luminal mucus (Aikawa *et al*., 1989).

The number of ciliated cells and the length of individual cilia is decreased in patients with chronic bronchitis (Wanner, 1977). Ciliary aberrations include compound cilia, cilia with an abnormal axoneme or intra-cytoplasmic microtubule doublets, and cilia enclosed within periciliary sheaths (McDowell *et al*., 1976). These abnormalities coupled with mucous hypersecretion are presumably associated with reduced mucus clearance and airway mucus obstruction in the bronchitic component of COPD.

7.7 Airway mucus hypersecretory phenotype in asthma

Asthma is a chronic inflammatory condition of the airway characterized by variable airflow limitation that is at least partially reversible, either spontaneously or with treatment (American Thoracic Society, 1987; British Thoracic Society, 1997). It has specific clinical and pathophysiological features (Eapen and Busse, 2002), including mucus obstruction of the airway (Rogers, 2004). The latter is particularly evident in a proportion of patients who die in *status asthmaticus*, where many airway are occluded by mucus plugs (Dunnill, 1960; Houston *et al*., 1953; Saetta *et al*., 1991). The plugs are highly viscous and comprise plasma proteins, DNA, cells, proteoglycans (Bhaskar *et al*., 1988) and mucins (Bhaskar *et al*., 1988; Dunnill, 1960; Sheehan *et al*., 1995). Incomplete plugs are found in the airway of asthmatic subjects who have died from causes other than asthma (Dunnill, 1975), which indicates that plug formation is a chronic, progressive process. There is also more mucus in the central and peripheral airway of both chronic and severe asthmatics compared with control subjects (Aikawa *et al*., 1992). Analysis of asthmatic sputum indicates that the mucus comprises DNA, lactoferrin, eosinophil cationic protein, and plasma proteins such as albumin and fibrinogen (Fahy *et al*., 1993b, 1993a; Lopez-Vidriero and Reid, 1978b), as well as mucins (Fahy *et al*., 1993b; Lopez-Vidriero and Reid, 1978a; Ordonez *et al*., 2001). The increased amount of luminal mucus reflects an increase in amount of airway secretory tissue, due to both goblet cell hyperplasia (Aikawa *et al*., 1992; Ordonez *et al*., 2001) and submucosal gland hypertrophy (Dunnill, 1960), although the latter is not characteristic of all patients with asthma (Aikawa *et al*., 1992).

Airway epithelial fragility, with epithelial shedding in extreme cases, is a significant feature of asthma (Bousquet *et al*., 2000). Shedding includes loss of ciliated cells, with presumably a concomitant reduction in mucus clearing capacity. As in COPD above, abnormalities in airway ciliated cells and cilia have been described in asthma. The ciliated cells themselves may be damaged, with loss of cilia, vacuolization of the endoplasmic reticulum and mitochondria, and microtubule damage (Beasley *et al*., 1989; Carson *et al*., 1994;

Laitinen *et al*., 1985). These abnormalities could be caused by some of the inflammatory mediators generated in the airway of asthmatic patients, for example eosinophil major basic protein (Gleich *et al*., 1983). A variety of other ciliostatic and ciliotoxic compounds are also present in asthmatic airway secretions (Del Donno *et al*., 2000).

In summary of this section, the combination of an increased amount of mucus-secreting tissue, with associated mucus hypersecretion, the production of viscid mucus, and abnormal ciliary function leads to reduced mucus clearance and the development of airway mucus obstruction in asthma.

7.8 Mucociliary clearance in asthma and COPD

Clearance of mucus from the airway is impaired in patients with a variety of respiratory diseases, including asthma and COPD (Wanner *et al*., 1996). However, it should be noted that there are often discrepancies in results between studies that are invariably due to differences in methodology (Clarke and Pavia, 1980; Pavia *et al*., 1983), but may also be due to observations made at different stages of disease.

7.8.1 COPD

Mucus clearance is generally considered to be impaired in patients with COPD (Wanner *et al*., 1996). However, the validity of these studies is dependent upon patient selection and the exclusion of patients with asthma. For example, patients classified as having obstructive chronic bronchitis and with a bronchial reversibility of less than 15 per cent had slower lung mucus clearance than patients with reversibility greater than 15 per cent and who, therefore, were likely to be asthmatic (Moretti *et al*., 1997). Nevertheless, mucus clearance is significantly reduced in heavy smokers (Goodman *et al*., 1978) and in patients with chronic bronchitis (Agnew *et al*., 1982). Lung mucus clearance differs between patients with chronic airway obstruction, with or without emphysema (van der Schans *et al*., 1990). Both groups of patients were smokers or ex-smokers and had productive cough, but lung elastic recoil pressure was reduced in the emphysema group. Mucus clearance from central airway was similar. In contrast, clearance from the peripheral lung was faster in the emphysema group than in the patients without emphysema. Importantly, forced expirations and cough markedly increased peripheral clearance in the non-emphysema group but not in the emphysema group. Comparable findings in a subsequent study led to the suggestion that cough compensates relatively effectively for decreased mucus clearance in patients with chronic bronchitis (Ericsson *et al*., 1995). Conversely, cough is not so effective in COPD patients with impaired lung elastic recoil.

7.8.2 Asthma

Airway mucociliary clearance is well documented as being impaired in asthma (Del Donno *et al*., 2000). Clearance is impaired even in patients in remission (Pavia *et al*., 1985) and in those with mild stable disease (Bateman *et al*., 1983). Mucus clearance is proportionally reduced in symptomatic asthmatics (Foster *et al*., 1982) and during exacerbations (Messina *et al*., 1991). In addition, the normal slowing of mucus clearance during sleep is more pronounced in asthmatic patients (Bateman *et al*., 1978; Pavia *et al*., 1987), and this could

be a contributory factor in nocturnal asthma. The mechanisms underlying the reduced mucus clearance in asthma are not clearly defined, although airway inflammation is considered to be a major contributor (Del Donno *et al*., 2000).

7.9 Mechanisms of airway goblet cell hyperplasia

Airway goblet cell hyperplasia is a predominant feature of COPD, asthma and CF (see above), and is an often-used end-point in animal models of respiratory disease (Rogers, 1997). The cellular composition of the airway epithelium can alter both by cell division and by differentiation of one cell into another (Ayers and Jeffery, 1988). There are at least eight cell types in the airway epithelium of the conducting airway. Of these, the basal, serous and Clara cells are considered progenitor cells with the capacity to undergo division followed by differentiation into 'mature' ciliated or goblet cells. In specific experimental conditions, for example exposure to cigarette smoke, goblet cell division contributes in part to the hyperplasia. However, differentiation of non-granulated airway epithelial cells is a major route for production of new goblet cells (Ayers and Jeffery, 1988; Nadel and Burgel, 2001; Rogers, 1994). In experimental animals, production of goblet cells is usually at the 'expense' of the progenitor cells, most notably serous and Clara cells, which decrease in number as goblet cell numbers increase. Serous-like cells and Clara cells are found in macroscopically normal bronchioles in human lung (Rogers *et al*., 1993). Whether there is a reduction in number in respiratory disease is not reported, but merits investigation. Reduction in the relative proportion of serous and Clara cells has pathophysiological significance because they produce a number of anti-inflammatory, immunomodulatory and antibacterial molecules vital to host defence (Basbaum *et al*., 1990; Singh and Katyal, 2000). For example, serous cells produce lysozyme, lactoferrin, secretory IgA, peroxidase and at least two protease inhibitors. Clara cells produce Clara cell 10-kDa protein, also known as uteroglobulin, Clara cell 55-kDa protein, Clara cell tryptase, β-galactoside-binding lectin, possibly a specific phospholipase, and surfactant proteins A, B, and D. Thus, in respiratory diseases associated with airway mucus hypersecretion it seems that not only is there goblet cell hyperplasia, with associated mucus hypersecretion, but also a reduction in serous and Clara cells, with concomitant impaired potential for host defence.

7.10 Differences in mucus hypersecretory phenotype between asthma, COPD and CF

In order to develop appropriate models of airway mucus obstruction and develop drugs to aid mucus clearance, it is necessary to understand the similarities and differences in the features of mucus obstruction for different hypersecretory conditions. There are a number of differences in the pathophysiology of impaired mucus clearance between asthma, COPD and CF (Figure 7.5). Firstly, although the underlying pulmonary inflammation of asthma and COPD shares many common features, there are specific characteristics unique to each condition (Djukanovic, 2002; Jeffery, 1999; Saetta *et al*., 2001). Asthma is almost invariably an allergic disease that affects the airway, rather than the lung parenchyma, and is characterized by Th2-lymphocyte orchestration of pulmonary eosinophilia. The reticular layer beneath the basement membrane is markedly thickened and the airway epithelium is fragile, features not

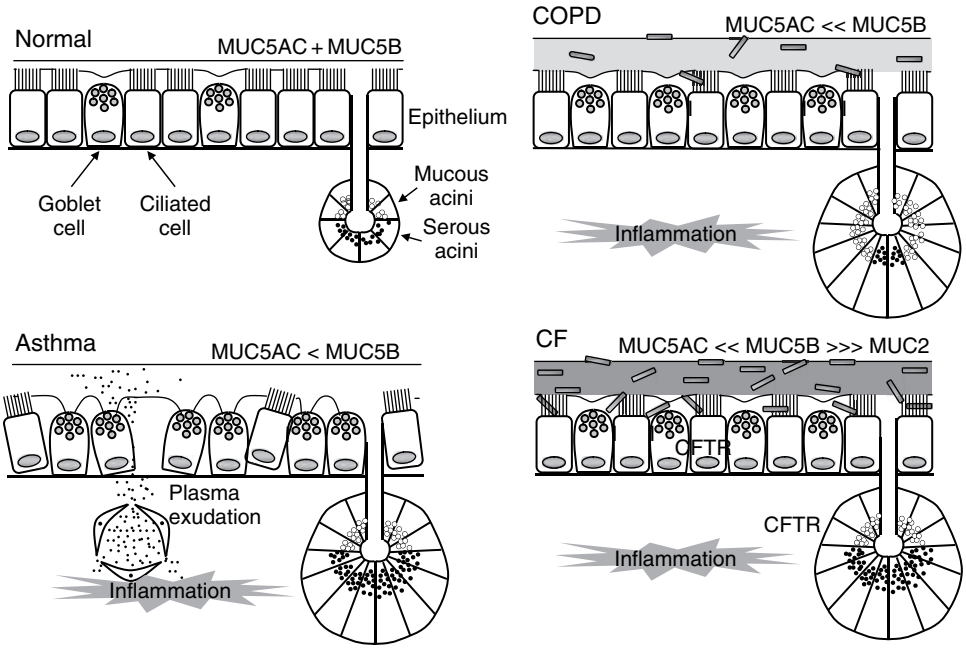


Figure 7.5 Putative differences in pathophysiology of airway mucus obstruction in COPD, asthma and CF. Compared with normal, in COPD, there is airway inflammation, increased luminal mucus, goblet cell hyperplasia, submucosal gland hypertrophy (with an increased proportion of mucous to serous acini), an increased ratio of mucin MUC5B to MUC5AC above that in asthma, and a susceptibility to infection. In asthma, there is airway inflammation, increased luminal mucus, with an increased ratio of MUC5B to MUC5AC, epithelial 'fragility' with loss of ciliated cells, marked goblet cell hyperplasia, submucosal gland hypertrophy (although without a marked increase in mucous to serous ratio), 'tethering' of mucus to goblet cells, and plasma exudation. In CF, there is airway inflammation, increased luminal mucus, goblet cell hyperplasia, submucosal gland hypertrophy, an increased ratio of MUC5B to MUC5AC, small amounts of MUC2 present in the mucus, and a marked susceptibility to infection. Many of these observations require confirmation (or otherwise) by data from greater numbers of subjects

usually associated with COPD. The bronchial inflammatory infiltrate comprises activated T-cells (predominantly $CD4^+$ cells) and eosinophils. Neutrophils are generally sparse in stable disease. In contrast, COPD is currently perceived as predominantly a neutrophilic disorder governed largely by macrophages and epithelial cells. It is associated primarily with cigarette-smoking. Three conditions comprise COPD, namely mucous hypersecretion, bronchiolitis and emphysema. The latter feature is not associated with asthma. In addition, and in contrast to asthma, $CD8^+$ T-lymphocytes predominate and pulmonary eosinophilia is generally associated with exacerbations.

Both asthma and COPD have a characteristic 'portfolio' of inflammatory mediators and enzymes, many of which differ between the two conditions (Barnes *et al*., 1998; Barnes, 2002). At a very simplified level, histamine, interleukin (IL)-4 and eotaxin are associated with asthma, whilst IL-8, neutrophil elastase and matrix metalloproteinases are associated with COPD. Thus, there are specific differences between asthma and COPD in their airway

inflammation and remodelling. These differences may in turn exert different influences on the development of airway mucus obstruction in the two conditions (Figure 7.2).

Airway mucus in asthma is more viscous than in COPD or CF, with the airway of asthmatic patients tending to develop, and subsequently become blocked by, gelatinous 'mucus' plugs (Liu *et al*., 1998). Whether or not mucus in asthma has an intrinsic biochemical abnormality is unclear. In general terms, sputum from patients with asthma is more viscous than that from patients with chronic bronchitis or bronchiectasis (Charman and Reid, 1972; Lopez-Vidriero and Reid, 1978b; Shimura *et al*., 1988). Mucus plugs in asthma differ from airway mucus gels in chronic bronchitis or CF in that they are stabilized by non-covalent interactions between extremely large mucins assembled from conventional-sized subunits (Sheehan *et al*., 1995). This suggests an intrinsic abnormality in the mucus due to a defect in assembly of the mucin molecules, and could account for the increased viscosity of the mucus plugs in asthma. Plug formation may also be due, at least in part, to increased airway plasma exudation in asthma compared with COPD (Rogers and Evans, 1992). In addition, in direct contrast to COPD, exocytosed mucins in asthma are not released fully from the goblet cells, leading to 'tethering' of luminal mucins to the airway epithelium (Shimura *et al*., 1996). This tethering may also contribute to plug formation. One explanation of mucus tethering is that proteases from neutrophils, the predominant inflammatory cell in COPD (Pauwels *et al*., 2001), cleave goblet cell-attached mucins. In asthma, the inflammatory cell profile, predominantly airway eosinophilia (Eapen and Busse, 2002), does not generate the appropriate proteases to facilitate mucin release.

Different MUC gene products, or at least different proportions of these mucins, appear to be present in respiratory tract secretions in COPD, asthma and CF. MUC5AC and a low-charge glycoform of MUC5B are the major mucin species in airway secretions from patients with COPD, asthma or CF (Hovenberg *et al*., 1996b; Kirkham *et al*., 2002; Sheehan *et al*., 1999; Thornton *et al*., 1996; Wickstrom *et al*., 1998). There is significantly more of the low-charge glycoform of MUC5B in the respiratory diseases than in normal control secretions (Kirkham *et al*., 2002). An interesting difference between the disease conditions is that there is a proportional increase in the MUC5B mucin over the MUC5AC mucin in airway secretions from patients with CF or COPD compared with secretions from patients with asthma (Thornton *et al*., 1996). These data above require confirmation in more samples. The significance of the change in MUC5B glycoforms between the different diseases in unclear. However, it may relate to differences in propensity of bacterial colonization of the lungs. It is noteworthy that it is COPD and CF, both diseases in which patients are prone to infection (Davis, 2001; Pauwels *et al*., 2001), that share the same proportional reduction in serous cells, rather than asthma, a condition in which patients are not so notably prone to chest infection.

In contrast to normal airway, goblet cells in the airway from patients with COPD contain not only MUC5AC but also MUC5B (Chen *et al*., 2001; Wickstrom *et al*., 1998) and MUC2 (Davies *et al*., 2002; Davies and Carlstedt, 2001). This distribution is different from that in the airway of patients with asthma or CF, where MUC5AC and MUC5B show a similar histological pattern to normal controls (Groneberg *et al*., 2002a, 2002b). It is noteworthy that although MUC2 is located in goblet cells in irritated airway, and MUC2 mRNA is found in the airway of smokers (Steiger *et al*., 1994), MUC2 mucin is either not found in airway secretions from normal subjects or patients with chronic bronchitis (Hovenberg *et al*., 1996b), or is found only in very small amounts in asthma, COPD or CF (Davies *et al*., 1999; Kirkham *et al*., 2002). The significance of the above combined observations is unclear, but suggests that there are differences in goblet cell phenotype between asthma, COPD and CF.

Another notable difference between asthma and COPD is in the bronchial submucosal glands (Glynn and Michaels, 1960). In asthma, although hypertrophied, the glands are morphologically normal with an even distribution of mucous and serous cells. In contrast, in chronic bronchitis, gland hypertrophy is characterized by a markedly increased number of mucous cells relative to serous cells, particularly in severe bronchitis. The reduction in number of gland serous cells may have clinical significance. The serous cells are a rich source of antibacterial proteins, such as lysozyme and lactoferrin (Finkbeiner, 1999). Thus, the airway mucus layer in COPD patients may have a reduced antibacterial capacity compared with that in asthma. This reduction, coupled with the change in MUC5B glycoforms in COPD (see above), could further explain, at least in part, the much higher incidence of bacterial chest infections in COPD compared with asthma.

Finally, it is not clear whether or not there are differences in the airway ciliary abnormalities between COPD, asthma or CF (see above). However, epithelial fragility and shedding are features of asthma rather than COPD (Jeffery, 1999), which suggests that there may be greater loss of, and damage to, the ciliated cells in asthma compared with COPD or CF.

From the above, it may be seen that there are theoretical and actual differences in the nature of airway mucus obstruction between COPD, asthma and CF. How these relate to pathophysiology and clinical symptoms in the three conditions is, for the most part, unclear. However, these dissimilarities indicate that different treatments are required for effective treatment of airway mucus obstruction in different respiratory diseases.

7.11 Conclusions

Production of airway mucus is a vital homeostatic mechanism that protects the respiratory tract from a barrage of inhaled insult. Precise interaction between cilia, the periciliary layer and mucus is required for optimal mucociliary clearance. However, abnormal production of mucus can contribute to respiratory disease. Airway obstruction by mucus is a common feature of a number of severe respiratory conditions, including asthma, COPD and CF. These diseases share pulmonary inflammation and remodelling as a pathophysiological characteristic. They also each have a number of unique features that characterize their airway mucus obstruction. For example, plasma exudation, mucus plug formation, and mucus tethering are features of asthma, whereas submucosal gland hypertrophy with a disproportionate increase in the ratio of mucous to serous cells is a significant feature in COPD. Understanding of the relative importance of the differences and similarities in the pathophysiology of the different mucus hypersecretory phenotypes between different respiratory diseases should lead to rational development of pharmacotherapeutic interventions. However, it should be noted that these interventions may have unexpected and unwanted side-effects. For example, airway goblet cell hyperplasia is a notable feature of COPD, asthma and CF (see above). Consequently, a reasonable therapeutic aim might be to inhibit or reverse the increase in goblet cell number. However, airway goblet cells, as well as producing mucins, produce a variety of anti-inflammatory and immunomodulatory molecules. For example, sheep goblet cells produce in abundance a lactoperoxidase that potently scavenges hydrogen peroxide, an important mediator of oxidative stress and associated inflammation (Forteza *et al*., 2001; Salathe *et al*., 1997), whilst hyperplastic goblet cells in a rat model of allergic asthma produce the anti-inflammatory and immunomodulatory molecule surfactant protein (SP)-D (Kasper, 2002). Thus, goblet cell secretions are a combination of mucins and host defence molecules.

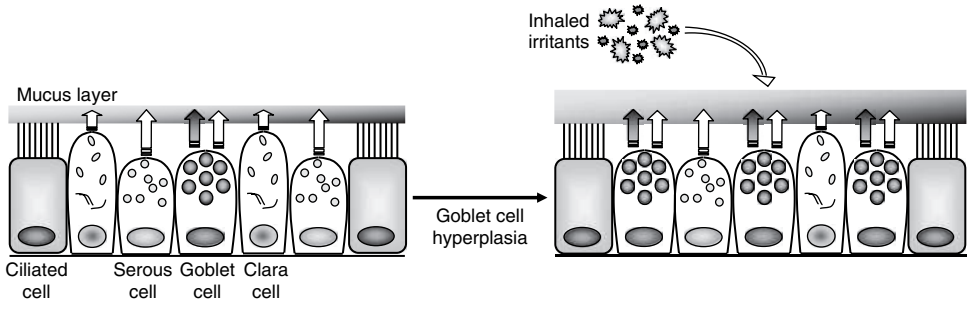


Figure 7.6 Hypothesis for airway goblet cell hyperplasia as a hypersecretory and anti-inflammatory process. Left-hand panel: Normal epithelium is covered by a thin layer of mucus and comprises ciliated, goblet, serous and Clara cells. Goblet cells secrete mucins (dark arrows) that contribute to formation of the mucus layer. Goblet cells, serous cells and Clara cells produce antibacterial, anti-inflammatory and immunomodulatory molecules (e.g. surfactant proteins, peroxidases and lysozyme) that contribute to airway defence (light arrows). Right-hand panel: Goblet cell hyperplasia. Inhaled irritants induce goblet cell hyperplasia, which leads to mucus hypersecretion and production of a thicker mucus layer (to protect the epithelium from inhaled 'insult'). The increased number of goblet cells is at the 'expense' of serous and Clara cells. However, goblet cells also produce host defence molecules, which means that the airway inflammatory 'shield' is balanced despite loss of serous and Clara cells

The implication of this is that the reduced anti-inflammatory 'shield', as a result of goblet cells replacing serous and Clara cells, is compensated for by the increase in host defence molecules in hyperplastic goblet cells (Figure 7.6). Consequently, any therapeutic intervention to reduce goblet cell number would presumably need to replace goblet cell-derived anti-inflammatory and immunomodulatory molecules to be entirely effective.

References

Agnew JE, Little F, Pavia D, and Clarke SW (1982) Mucus clearance from the airways in chronic bronchitis – Smokers and ex-smokers. *Bull Eur Physiopathol Respir*, **18**, 473–484.

Aikawa T, Shimura S, Sasaki H, Takishima T, Yaegashi H, and Takahashi T (1989) Morphometric analysis of intraluminal mucus in airways in chronic obstructive pulmonary disease. *Am Rev Respir Dis*, **140**, 477–482.

Aikawa T, Shimura S, Sasaki H, Ebina M, and Takishima T (1992) Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest*, **101**, 916–921.

American Thoracic Society (1987) Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis*, **136**, 225–244.

Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, and Aubert JP (1993) Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. *J Histochem Cytochem*, **41**, 1479–1485.

Ayers MM and Jeffery PK (1988) Proliferation and differentiation in mammalian airway epithelium. *Eur Respir J*, **1**, 58–80.

Barnes PJ (2002) New treatments for COPD. *Nat Rev Drug Discov*, **1**, 437–446.

Barnes PJ, Chung KF, and Page CP (1998) Inflammatory mediators of asthma: an update. *Pharmacol Rev*, **50**, 515–596.

Basbaum CB, Jany B, and Finkbeiner WE (1990) The serous cell. *Annu Rev Physiol*, **52**, 97–113.

Bateman JR, Pavia D, and Clarke SW (1978) The retention of lung secretions during the night in normal subjects. *Clin Sci Mol Med Suppl*, **55**, 523–527.

Bateman JR, Pavia D, Sheahan NF, Agnew JE, and Clarke SW (1983) Impaired tracheobronchial clearance in patients with mild stable asthma. *Thorax*, **38**, 463–467.

Beasley R, Roche WR, Roberts JA, and Holgate ST (1989) Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis*, **139**, 806–817.

Bhaskar KR, O'Sullivan DD, Coles SJ, Kozakewich H, Vawter GP, and Reid LM (1988) Characterization of airway mucus from a fatal case of status asthmaticus. *Pediatr Pulmonol*, **5**, 176–182.

Bousquet J, Jeffery PK, Busse WW, Johnson M, and Vignola AM (2000) Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med*, **161**, 1720–1745.

British Thoracic Society (1997) The British guidelines on asthma management. *Thorax*, **52** (Suppl 1), S1–S21.

Carson JL, Collier AM, Fernald GW, and Hu SC (1994) Microtubular discontinuities as acquired ciliary defects in airway epithelium of patients with chronic respiratory diseases. *Ultrastruct Pathol*, **18**, 327–332.

Charman J and Reid L (1972) Sputum viscosity in chronic bronchitis, bronchiectasis, asthma and cystic fibrosis. *Biorheology*, **9**, 185–199.

Chen Y, Zhao YH, Di YP, and Wu R (2001) Characterization of human mucin 5B gene expression in airway epithelium and the genomic clone of the amino-terminal and 5′-flanking region. *Am J Respir Cell Mol Biol*, **25**, 542–553.

Clarke SW and Pavia D (1980) Lung mucus production and mucociliary clearance: methods of assessment. *Br J Clin Pharmacol*, **9**, 537–546.

Davies JR and Carlstedt I (2001) Respiratory tract mucins. In: *Cilia and Mucus: From Development to Respiratory Defense*, M. Salathe (ed.), pp. 167–178, Marcel Dekker, New York.

Davies JR, Svitacheva N, Lannefors L, Kornfalt R, and Carlstedt I (1999) Identification of MUC5B, MUC5AC and small amounts of MUC2 mucins in cystic fibrosis airway secretions. *Biochem J*, **344** (Pt 2), 321–330.

Davies JR, Herrmann A, Russell W, Svitacheva N, Wickström C, and Carlstedt I (2002) Respiratory tract mucins: structure and expression patterns. *Novartis Found Symp*, **248**, 76–88.

Davis PB (2001) Cystic fibrosis. *Pediatr Rev*, **22**, 257–264.

Del Donno M, Bittesnich D, Chetta A, Olivieri D, and Lopez-Vidriero MT (2000) The effect of inflammation on mucociliary clearance in asthma: an overview. *Chest*, **118**, 1142–1149.

Dell A and Morris HR (2001) Glycoprotein structure determination by mass spectrometry. *Science*, **291**, 2351–2356.

Desseyn JL, Buisine MP, Porchet N, Aubert JP, and Laine A (1998) Genomic organization of the human mucin gene MUC5B. cDNA and genomic sequences upstream of the large central exon. *J Biol Chem*, **273**, 30157–30164.

Djukanovic R (2002) Airway inflammation in asthma and its consequences: implications for treatment in children and adults. *J Allergy Clin Immunol*, **109**, S539–S548.

Dunnill MS (1960) The pathology of asthma with special reference to changes in the bronchial mucosa. *J Clin Pathol*, **13**, 27–33.

Dunnill MS (1975) The morphology of the airways in bronchial asthma. In: *New Directions in Asthma*, M. Stein (ed.), pp. 213–221, American College of Physicians, Park Ridge IL.

Eapen SS and Busse WW (2002) Asthma. *Clin Allergy Immunol*, **16**, 325–353.

Ericsson CH, Svartengren K, Svartengren M, Mossberg B, Philipson K, Blomquist M, and Camner P (1995) Repeatability of airway deposition and tracheobronchial clearance rate over three days in chronic bronchitis. *Eur Respir J*, **8**, 1886–1893.

Fahy JV, Liu J, Wong H, and Boushey HA (1993a) Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis*, **147**, 1126–1131.

Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, and Boushey HA (1993b) Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis*, **147**, 1132–1137.

Finkbeiner WE (1999) Physiology and pathology of tracheobronchial glands. *Respir Physiol*, **118**, 77–83.

Forteza R, Lieb T, Aoki T, Savani RC, Conner GE, and Salathe M (2001) Hyaluronan serves a novel role in airway mucosal host defense. *FASEB J*, **15**, 2179–2186.

Foster WM, Langenback EG, and Bergofsky EH (1982) Lung mucociliary function in man: interdependence of bronchial and tracheal mucus transport velocities with lung clearance in bronchial asthma and healthy subjects. *Ann Occup Hyg*, **26**, 227–244.

Gleich GJ, Loegering DA, Frigas E, and Filley WV (1983) The eosinophil granule major basic protein: biological activities and relationship to bronchial asthma. *Monogr Allergy*, **18**, 277–283.

Glynn AA and Michaels L (1960) Bronchial biopsy in chronic bronchitis and asthma. *Thorax*, **15**, 142–153.

Global Initiative for Chronic Obstructive Lung Disease (2006) Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. www.goldcopd.org.

Goodman RM, Yergin BM, Landa JF, Golivanux MH, and Sackner MA (1978) Relationship of smoking history and pulmonary function tests to tracheal mucous velocity in nonsmokers, young smokers, ex-smokers, and patients with chronic bronchitis. *Am Rev Respir Dis*, **117**, 205–214.

Groneberg DA, Eynott PR, Lim S, Oates T, Wu R, Carlstedt I, Roberts P, McCann B, Nicholson AG, Harrison BD, and Chung KF (2002a) Expression of respiratory mucins in fatal status asthmaticus and mild asthma. *Histopathology*, **40**, 367–373.

Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, Nicholson AG, and Chung KF (2002b) Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. *Respir Med*, **96**, 81–86.

Guyonnet Duperat, V, Audie JP, Debailleul V, Laine A, Buisine MP, Galiegue-Zouitina S, Pigny P, Degand P, Aubert JP, and Porchet N (1995) Characterization of the human mucin gene MUC5AC: a consensus cysteine-rich domain for 11p15 mucin genes? *Biochem J*, **305**, 211–219.

Hanisch FG (2001) O-glycosylation of the mucin type. *Biol Chem*, **382**, 143–149.

Hollander W and Stober W (1986) Aerosols of smoke, respiratory physiology and deposition. *Arch Toxicol Suppl*, **9**, 74–87.

Houston JC, De Navasquez S, and Trounce JR (1953) A clinical and pathological study of fatal cases of status asthmaticus. *Thorax*, **8**, 207–213.

Houtmeyers E, Gosselink R, Gayan-Ramirez G, and Decramer M (1999) Regulation of mucociliary clearance in health and disease. *Eur Respir J*, **13**, 1177–1188.

Hovenberg HW, Davies JR, and Carlstedt I (1996a) Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. *Biochem J*, **318**, 319–324.

Hovenberg HW, Davies JR, Herrmann A, Linden CJ, and Carlstedt I (1996b) MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. *Glycoconj J*, **13**, 839–847.

Jeffery PK (1999) Differences and similarities between chronic obstructive pulmonary disease and asthma. *Clin Exp Allergy*, **29** (Suppl 2), 14–26.

Kasper M, Sims G, Koslowski R, Kuss H, Thuemmler M, Fehrenbach H, and Auten RL. (2002) Increased surfactant protein D (SP-D) in rat airway goblet and Clara cells during ovalbumin induced allergic airway inflammation. *Clin Exp Allergy*, **32**, 1251–1258.

Kirkham S, Sheehan JK, Knight D, Richardson PS, and Thornton DJ (2002) Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J*, **361**, 537–546.

Knowles MR and Boucher RC (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest*, **109**, 571–577.

Laitinen LA, Heino M, Laitinen A, Kava T, and Haahtela T (1985) Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis*, **131**, 599–606.

Lippmann M, Yeates DB, and Albert RE (1980) Deposition, retention, and clearance of inhaled particles. *Br J Ind Med*, **37**, 337–362.

Liu YC, Khawaja AM, and Rogers DF (1998) Pathophysiology of airway Mucus Secretion in asthma. In: *Asthma: Basic Mechanics and Clinical Management*, 3rd edn. Batnes P, Rodger I, and Thomson N (eds). Elsevier, Oxford. **Third**, 205–227.

Lopez-Vidriero MT and Reid L (1978a) Bronchial mucus in health and disease. *Br Med Bull*, **34**, 63–74.

Lopez-Vidriero MT and Reid L (1978b) Chemical markers of mucous and serum glycoproteins and their relation to viscosity in mucoid and purulent sputum from various hypersecretory diseases. *Am Rev Respir Dis*, **117**, 465–477.

Maestrelli P, Saetta M, Mapp CE, and Fabbri LM (2001) Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, **164**, S76–S80.

McDowell EM, Barrett LA, Harris CC, and Trump BF (1976) Abnormal cilia in human bronchial epithelium. *Arch Pathol Lab Med*, **100**, 429–436.

Messina MS, O'Riordan TG, and Smaldone GC (1991) Changes in mucociliary clearance during acute exacerbations of asthma. *Am Rev Respir Dis*, **143**, 993–997.

Moniaux N, Escande F, Porchet N, Aubert JP, and Batra SK (2001) Structural organization and classification of the human mucin genes. *Front Biosci*, **6**, D1192–D1206.

Moretti M, Lopez-Vidriero MT, Pavia D, and Clarke SW (1997) Relationship between bronchial reversibility and tracheobronchial clearance in patients with chronic bronchitis. *Thorax*, **52**, 176–180.

Mullen JB, Wright JL, Wiggs BR, Pare PD, and Hogg JC (1987) Structure of central airways in current smokers and ex-smokers with and without mucus hypersecretion: relationship to lung function. *Thorax*, **42**, 843–848.

Nadel JA and Burgel PR (2001) The role of epidermal growth factor in mucus production. *Curr Opin Pharmacol*, **1**, 254–258.

Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, and Fahy JV (2001) Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med*, **163**, 517–523.

Pauwels R, Buist AS, Calverley PMA, Jenkins CR, and Hurd SS. (2001). Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global initiative for chronic onstructive lung disease (GOLD) workshop summary. *Am J Respir Crit Care Med* **163**, 1256–1276.

Pavia D, Sutton PP, Agnew JE, Lopez-Vidriero MT, Newman SP, and Clarke SW (1983) Measurement of bronchial mucociliary clearance. *Eur J Respir Dis Suppl*, **127**, 41–56.

Pavia D, Bateman JR, Sheahan NF, Agnew JE, and Clarke SW (1985) Tracheobronchial mucociliary clearance in asthma: impairment during remission. *Thorax*, **40**, 171–175.

Pavia D, Lopez-Vidriero MT, and Clarke SW (1987) Mediators and mucociliary clearance in asthma. *Bull Eur Physiopathol Respir*, **23** (Suppl 10), 89s–94s.

Reid CJ, Gould S, and Harris A (1997) Developmental expression of mucin genes in the human respiratory tract. *Am J Respir Cell Mol Biol*, **17**, 592–598.

Reid L (1954) Pathology of chronic bronchitis. *Lancet*, **266**, 275–278.

Reid L (1960) Measurement of the bronchial mucous gland layer: a diagnostis yardstick in chronic bronchitis. *Thorax*, **15**, 132–141.

Restrepo G and Heard BE (1963) The size of the bronchial glands in chronic bronchitis. *J Pathol Bacteriol*, **85**, 305–310.

Robinson M and Bye PT (2002) Mucociliary clearance in cystic fibrosis. *Pediatr Pulmonol*, **33**, 293–306.

Rogers AV, Dewar A, Corrin B, and Jeffery PK (1993) Identification of serous-like cells in the surface epithelium of human bronchioles. *Eur Respir J*, **6**, 498–504.

Rogers DF (1994) Airway goblet cells: responsive and adaptable front-line defenders. *Eur Respir J*, **7**, 1690–1706.

Rogers DF (1997) In vivo preclinical test models for studying airway mucus secretion. *Pulm Pharmacol Ther*, **10**, 121–128.

Rogers DF (2003) The airway goblet cell. *Int J Biochem Cell Biol*, **35**, 1–6.

Rogers DF (2004) Airway mucus hypersecretion in asthma: an undervalued pathology? *Curr Opin Pharmacol*, **4**, 241–250.

Rogers DF and Evans TW (1992) Plasma exudation and oedema in asthma. *Br Med Bull*, **48**, 120–134.

Rose MC and Voynow JA (2006) Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev*, **86**, 245–278.

Rose MC, Nickola TJ, and Voynow JA (2001) Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. *Am J Respir Cell Mol Biol*, **25**, 533–537.

Saetta M, Di Stefano A, Rosina C, Thiene G, and Fabbri LM (1991) Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *Am Rev Respir Dis*, **143**, 138–143.

Saetta M, Turato G, Baraldo S, Zanin A, Braccioni F, Mapp CE, Maestrelli P, Cavallesco G, Papi A, and Fabbri LM (2000) Goblet cell hyperplasia and epithelial inflammation in peripheral airways of smokers with both symptoms of chronic bronchitis and chronic airflow limitation. *Am J Respir Crit Care Med*, **161**, 1016–1021.

Saetta M, Turato G, Maestrelli P, Mapp CE, and Fabbri LM (2001) Cellular and structural bases of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, **163**, 1304–1309.

Salathe M, Holderby M, Forteza R, Abraham WM, Wanner A, and Conner GE (1997) Isolation and characterization of a peroxidase from the airway. *Am J Respir Cell Mol Biol*, **17**, 97–105.

Seaton A, MacNee W, Donaldson K, and Godden D (1995) Particulate air pollution and acute health effects. *Lancet*, **345**, 176–178.

Sharma P, Dudus L, Nielsen PA, Clausen H, Yankaskas JR, Hollingsworth MA, and Engelhardt JF (1998) MUC5B and MUC7 are differentially expressed in mucous and serous cells of submucosal glands in human bronchial airways. *Am J Respir Cell Mol Biol*, **19**, 30–37.

Sheehan JK, Thornton DJ, Somerville M, and Carlstedt I (1991) Mucin structure. The structure and heterogeneity of respiratory mucus glycoproteins. *Am Rev Respir Dis*, **144**, S4–S9.

Sheehan JK, Richardson PS, Fung DC, Howard M, and Thornton DJ (1995) Analysis of respiratory mucus glycoproteins in asthma: a detailed study from a patient who died in status asthmaticus. *Am J Respir Cell Mol Biol*, **13**, 748–756.

Sheehan JK, Howard M, Richardson PS, Longwill T, and Thornton DJ (1999) Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug. *Biochem J*, **338**, 507–513.

Shimura S, Sasaki T, Sasaki H, Takishima T, and Umeya K (1988) Viscoelastic properties of bronchorrhoea sputum in bronchial asthmatics. *Biorheology*, **25**, 173–179.

Shimura S, Andoh Y, Haraguchi M, and Shirato K (1996) Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma. *Eur Respir J*, **9**, 1395–1401.

Singh G and Katyal SL (2000) Clara cell proteins. *Ann N Y Acad Sci*, **923**, 43–58.

Sleigh MA, Blake JR, and Liron N (1988) The propulsion of mucus by cilia. *Am Rev Respir Dis*, **137**, 726–741.

Steiger D, Fahy J, Boushey H, Finkbeiner WE, and Basbaum C (1994) Use of mucin antibodies and cDNA probes to quantify hypersecretion in vivo in human airways. *Am J Respir Cell Mol Biol*, **10**, 538–545.

Thornton DJ, Davies JR, Kraayenbrink M, Richardson PS, Sheehan JK, and Carlstedt I (1990) Mucus glycoproteins from 'normal' human tracheobronchial secretion. *Biochem J*, **265**, 179–186.

Thornton DJ, Sheehan JK, and Carlstedt I (1991) Heterogeneity of mucus glycoproteins from cystic fibrotic sputum. Are there different families of mucins? *Biochem J*, **276**, 677–682.

Thornton DJ, Devine PL, Hanski C, Howard M, and Sheehan JK (1994) Identification of two major populations of mucins in respiratory secretions. *Am J Respir Crit Care Med*, **150**, 823–832.

Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, and Sheehan JK (1996) Respiratory mucins: identification of core proteins and glycoforms. *Biochem J*, **316**, 967–975.

Thornton DJ, Howard M, Khan N, and Sheehan JK (1997) Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule. *J Biol Chem*, **272**, 9561–9566.

van der Schans CP, Piers DA, Beekhuis H, Koeter GH, van der Mark TW, and Postma DS (1990) Effect of forced expirations on mucus clearance in patients with chronic airflow obstruction: effect of lung recoil pressure. *Thorax*, **45**, 623–627.

Wanner A (1977) Clinical aspects of mucociliary transport. *Am Rev Respir Dis*, **116**, 73–125.

Wanner A, Salathe M, and O'Riordan TG (1996) Mucociliary clearance in the airways. *Am J Respir Crit Care Med*, **154**, 1868–1902.

Wickstrom C, Davies JR, Eriksen GV, Veerman EC, and Carlstedt I (1998) MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. *Biochem J*, **334**, 685–693.

8

The Pulmonary Surfactant System

Nicole A. Rocca, Ruud A.W. Veldhuizen and **James F. Lewis**
The Lawson Health Research Institute, The University of Western Ontario, Canada

8.1 Overview

Pulmonary surfactant exists as a lipoprotein complex within alveoli and is essential for normal lung homeostasis. Surfactant serves two main functions; to decrease the work of breathing by reducing surface tension at the air–liquid interface, and to contribute to the host's innate defence system within the alveolar environment. In this chapter we will discuss the composition of surfactant, the intracellular and extracellular metabolism of surfactant, and review both the biophysical and immuno-modulatory functions of this material. Finally, we will discuss alterations to the surfactant system in lung disease and the functional consequences of these changes in conditions such as acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS), as well as the future potential use of exogenous surfactant administration as a possible treatment for patients with lung injury.

8.2 Surfactant Composition

Surfactant is a lipoprotein complex synthesized and secreted by type II alveolar epithelial cells. Pulmonary surfactant, which can be obtained from the lung for analyses via lung lavage, consists of approximately 90 per cent lipid and 10 per cent surfactant associated proteins, designated SP-A, B, C, and D (Goerke, 1998; Persson *et al*., 1989; Possmayer, 1988). The composition of surfactant, as described below and represented schematically in Figure 8.1, is remarkably similar among mammalian species as determined from lavage analyses from humans, bovine and rat species (Shelley *et al*., 1984). Within the lipid component of surfactant, approximately 85 per cent consists of phospholipids (PL), which are amphipathic, meaning that these molecules have both hydrophobic and hydrophilic components with class determining polar head groups and fatty acyl tails. The most abundant PL species is phosphatidylcholine ($\sim$60 per cent), of which 30–50 per cent is comprised of the disaturated species dipalmitoylphosphatidylcholine (DPPC). The second most abundant

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

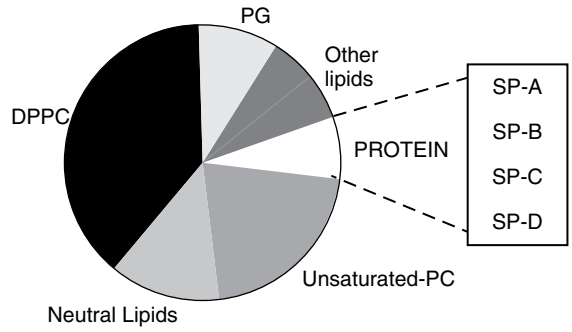


Figure 8.1 Schematic representation illustrating the major surfactant components in their relative proportions

PL is phosphatidylglycerol (PG), which makes up 7–15 per cent of total PL. The remaining PL components are minor compounds such as phosphatidylinositol, phosphatidylserine, sphingomyelin, and phosphatidylethanolamine. In addition to the phospholipids, surfactant also contains approximately 5 per cent neutral lipids; the majority of which is cholesterol (Veldhuizen and Possmayer 2004). Although the phospholipid profile is relatively conserved among species, it should be noted that some differences in composition are present within the fatty acid acyl chains of the specific phospholipids. Specific discussion of these differences, and their implications for surfactant function, is beyond the scope of this chapter, but is reviewed in detail by Postle *et al*. (2001).

The four surfactant-associated proteins (SP) represent approximately 10 per cent of total surfactant by weight. SP-A, SP-B, SP-C, and SP-D can be classified into two general categories: (1) the small hydrophobic proteins SP-B, and SP-C, and (2) the two larger multimeric, hydrophilic glycoproteins SP-A, and SP-D (Haagsman and Diemel, 2001; Hawgood and Shiffer, 1991). Both SP-B and SP-C are tightly associated with the lipids of surfactant and are predominantly formed within type II cells (Yu *et al*., 1987). In fact, these proteins remain associated with these lipids even during extraction with an organic solvent such as chloroform. SP-B is present within the lung as an 18 kDa homodimer (Hawgood *et al*., 1998). Although, SP-C is smaller, it is the most hydrophobic protein within the mammalian proteome and exists as a 4.2 kDa monomer. Adding to the hydrophobicity of this protein are the two palmitoylated adjacent cystine residues near the N-terminus (Curstedt *et al*., 1990; Johansson 1998).

The two remaining surfactant associated proteins SP-A and SP-D are both large hydrophilic proteins that have been identified as members of the collagen-like lectin (collectins) superfamily of immuno-modulatory proteins (Crouch *et al*., 2000; Kuroki and Sano, 1999; McCormack and Whitsett, 2002). These proteins share collagen-like triple helical sections linked to calcium-dependent regulatory domains that share structure with a number of mammalian lectins. The SP-A monomer is a 26- to 36-kDa protein synthesized within type II cells and Clara cells. SP-A is present within the lung as an octodecamer made up of six trimers in a ‘flower bouquet’ shape (Haagsman *et al*., 1987; McCormack, 1998). The SP-D monomer is a 42-kDa protein, but is typically found in a multimeric, cruciform structure within the lung (Crouch, 1998). Apart from being expressed in conducting airway (Clara cells) and alveolar type II cells in the lung, SP-D is also found in various other tissues

and cell types throughout the body, predominantly in mucosal surfaces such as the intestinal wall and nasal passages (Crouch, 1998). In contrast to SP-A, however, the majority of SP-D in fluid isolated via alveolar lavage is unassociated, or weakly associated, with surfactant lipids.

8.3 Surfactant Function

8.3.1 Biophysical function – physiological implications

The primary function of surfactant is to reduce surface tension forces exerted by water molecules at the air–liquid interface within the alveoli of the lung. While molecules in the bulk phase of the water layer experience equal attractive forces from other water molecules and thus exert a net force of zero within the liquid, surface tension arises at the interface. This is due to the unequal attractive forces between water and air molecules, whereby a net 'downward' force exists on the water molecules at the air–liquid interface. This unequal force on water molecules at the respiratory surface results in a cohesive surface layer forming the smallest surface area possible. Indeed surface tension accounts for approximately two-thirds of the contractile properties of the lungs, with the elastic tissue properties representing the remaining factor contributing to contraction. The physiological relevance of these forces can be explained by Laplace's Law which states that the pressure (ΔP) across the surface of a spherical structure (i.e. the alveolus) is equal to two times the tension (2γ) at the surface divided by the radius (r) of the sphere ($\Delta P = 2\gamma/r$). Therefore, the effort (or pressure) required to open a very small sphere (such as an alveolus prior to inhalation) would be extremely great if the surface tension values of the alveolus were high. Furthermore, given this relationship, the impact of surface tension on respiratory effort (or ΔP) is greater for alveoli with smaller radii, which would tend to completely collapse at the end of exhalation if surface tension did not change in accordance with respiration. This collapse and subsequent 'tearing open' of alveoli upon inhalation damages the epithelium of these distal airway, resulting in lung injury and impaired lung function. To resolve this issue, pulmonary surfactant is synthesized and secreted by alveolar type II cells and subsequently adsorbs to and spreads along the air–liquid interface. There, it displaces the water molecules present at the interface and prevents them from experiencing a net attractive force into the bulk phase, thus dramatically reducing the surface tension within the alveolus (see Figure 8.2). This reduction in surface tension confers stability to the alveolus, thus preventing alveolar collapse and reducing the work required to breathe. This is especially apparent during the exhalation phase of respiration, when alveolar radii are decreasing. It is during this decreasing radius of the alveolus, that surfactant molecules are compressed and the surface film 'purified', thus rendering it more effective at reducing surface tension. As a result, surface tension values are reduced in accordance with decreasing alveolar radius to prevent collapse at low lung volumes (see Figure 8.3).

The clinical relevance of these physiological concepts is reflected in the reduced lung function observed when there is a primary deficiency of surfactant. In humans, premature birth maybe associated with surfactant deficiency, as first described by Mary Allen Avery (Avery, 2000; Avery and Mead, 1959). This condition is termed 'neonatal respiratory distress syndrome' (NRDS) and has been successfully treated and prevented via the administration of exogenous surfactant, as will be discussed in more detail subsequently.

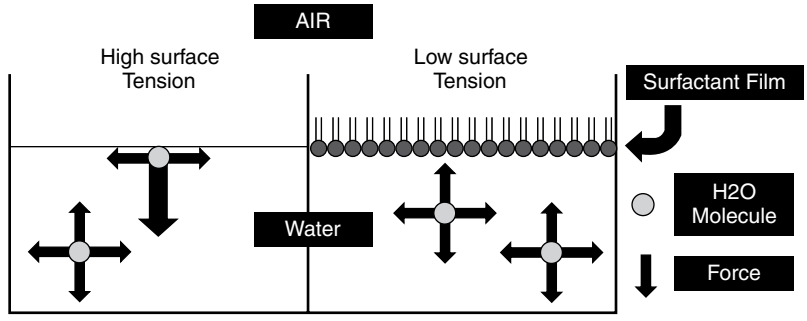


Figure 8.2 Air–liquid interface. (A) Interface without surfactant surface film. Water molecules (represented as the grey spheres) at the air–liquid interface experience a net downward force into the bulk phase of the water hypophase, as indicated by the large black arrow, creating high surface tension. (B) Interface with surfactant surface film (circles with two parallel lines represent the surfactant phospholipids). Displaced water molecules experience no net attractive forces, as indicated by equivalent arrows in all four directions, and thus surface tension is low

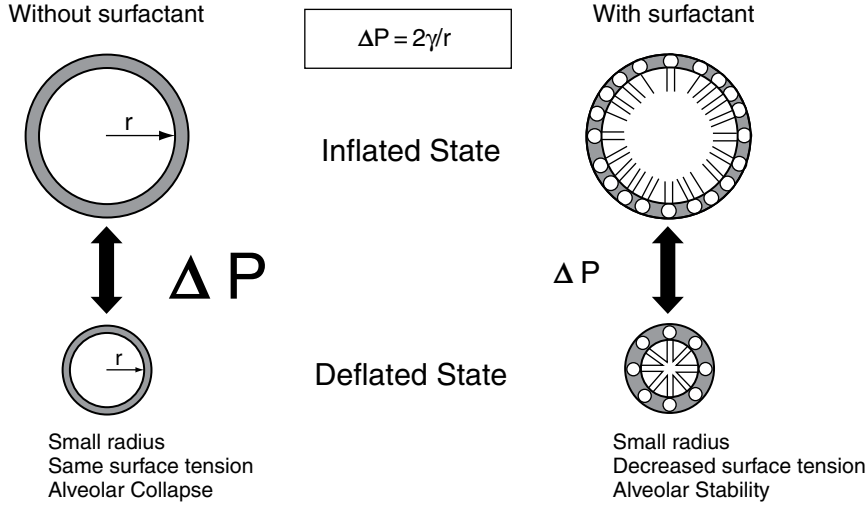


Figure 8.3 Schematic representation of the Law of LaPlace. $\Delta P =$ change in pressure across the sphere, $\gamma =$ surface tension, and $r =$ sphere radius. (A) The black line represents the epithelial layer of an individual alveolus, the grey layer is representative of the aqueous hypophase and (*r*) indicates the radius of the air space. As the radius of an alveolus without surfactant decreases, the surface tension remains the same and the change in pressure across the alveolus is great, thus leading to alveolar collapse. (B) The presence of a surfactant film (circles with two parallel lines indicate the surfactant phospholipids) reduces surface tension in accordance with decreasing radius, which stabilizes the pressure across the alveolus and maintains a patent airspace

An animal model of surfactant deficiency can be induced by repetitive whole lung saline lavage of adult animals or via premature delivery of pregnant animals. (Brackenbury *et al*., 2002; Yamada *et al*., 1990). In these models, reduced lung compliance and low blood

oxygenation is observed, similar to infants with NRDS. Maintaining appropriate blood oxygenation, and consequently survival, is dependent on the use of mechanical ventilation (MV), although outcomes are not always favourable. Administration of a biophysically active exogenous surfactant preparation into these surfactant-deficient lungs is very effective at restoring lung function. Thus, both human observations and animal experimental modelling indicate that surfactant is essential for normal lung function.

8.3.2 Biophysical function – molecular mechanisms

The molecular mechanisms by which surfactant reduces the surface tension at the air–liquid interface have been extensively studied. A variety of techniques, including the pulsating and captive bubble surfactometers, have been specifically developed to test the surface tension reducing activity of surfactant preparations in vitro (Enhorning, 1977; Schurch *et al*., 1989). Utilizing these techniques in combination with in vivo experiments, involving reconstitution studies using purified surfactant proteins and lipids in various combinations, has led to a better understanding of the role of each of the specific surfactant components in surface film formation and surface tension reduction (Veldhuizen and Haagsman, 2000).

The main components of surfactant responsible for surface tension reduction are the phospholipids. When spread at an air–liquid interface these amphipathic molecules align themselves with their polar head group in the aqueous phase and their hydrophobic acylchains projecting into the air. This effectively displaces water molecules at the surface and, with sufficient phospholipids present, reduces the surface tension to an equilibrium value of approximately 23 mN/m. Depending on the specific composition of the phospholipid monolayer, subsequent compression of the film can further reduce the surface tension to extremely low values. For example, it has been shown that monolayers containing the main component of surfactant lipid, DPPC, can be compressed and achieve surface tension values near to zero, as would be required in the lung. These findings, and the abundance of DPPC in natural surfactant suggest that the surfactant film reduces surface tension, in part, by forming monolayers of DPPC within the lung (Yu and Possmayer, 1992). Although other components of surfactant appear to be responsible for aiding in the purification of this lipid film of DPPC during the compression phase (Veldhuizen *et al*., 2000), over the last few years, it has become obvious that this concept of a monolayer is an oversimplification.

Two aspects of the proposed monolayer-model have been recently revised. First, several lines of evidence, including electron microscopic studies of the surface film within the lung tissue have demonstrated that the surface film is, in fact, a bilayer, or in some areas a multilayer (Bachofen *et al*., 2005; Bastacky *et al*., 1995; Schurch *et al*., 1995). The multiple layers of surfactant present underneath the surface film layer represent what has been called the ‘surface associated reservoir’ (Schurch *et al*., 1995). In vitro studies suggest this material can be rapidly inserted into the surface film upon expansion of the alveolar surface. It has also become clear that direct evidence for the proposed enrichment in DPPC within the surface film is lacking, and recent studies using techniques to image the surface of the film indicate that it may be the organization of the different lipids within surfactant that allows for surface tension be reduced to achieve low values (Piknova *et al*., 2001, 2002). This latter concept requires further study.

The role of the surfactant proteins in the above processes can be described in terms of the film formation, stabilization and maintenance. For example, when added to purified lipids, both SP-B and SP-C enhance the speed of the formation of a surface film, suggesting

that both proteins play a role in the adsorption of lipids to the air–liquid interface (Yu and Possmayer, 1990, 1992). SP-B also enhances surface tension reduction during film compression thereby stabilizing the surface film. Some in vitro studies have demonstrated a similar activity for SP-C, although to a lesser extent than SP-B. SP-C is believed to be more important in formation of the surface-associated reservoir by embedding in one bilayer with the two palmitic acids anchoring that bilayer to a parallel bilayer (Qanbar & Possmayer 1995).

Of the two hydrophilic proteins, only SP-A has been reported to contribute to the biophysical role of surfactant (Cockshutt *et al*., 1990). Although adding SP-A to pure surfactant phospholipids does not enhance surfactant function, in the presence of SP-B and phospholipids, SP-A does enhance the formation of a surface film. Furthermore, in vitro studies have shown that SP-A increases the surface tension reducing activity of surfactant at low concentrations, and in the presence of inhibitory substances such as serum proteins (Cockshutt *et al*., 1990; Rodriguez-Capote *et al*., 2003; Strayer *et al*., 1996). These observations suggest that SP-A may be more important biophysically, in situations in which the surfactant system is compromised, such as lung injury, rather than normal lung homeostasis.

In addition to the in vitro studies, information supporting the important role of surfactant proteins in lung function has also been obtained from knockout animals (Weaver and Beck, 1999). The most severe phenotypic abnormality observed in this setting is the SP-B knockout model. These animals died from severe respiratory failure shortly after birth (Tokieda *et al*., 1997). This provides strong evidence for the critical role of SP-B for surfactant function and, indeed, lung function in vivo. SP-C-deficient animals also have pulmonary phenotypic abnormalities; however, these abnormalities appear to be dependent on the specific genetic strain of the mice (Glasser *et al*., 2001, 2003). Although this intriguing finding requires further study, at this stage it can be assumed that SP-C is not essential for surfactant function in murine lungs. As suggested above, SP-A knockout animals are phenotypically normal under stable conditions (Korfhagen *et al*., 1998), although this is not the case in stressed situations, as will be described in more detail in subsequent sections.

8.3.3 Innate host defence

The respiratory system, with its continuous exposure to the external environment, is constantly subjected to inhaled or ingested pathogens, particles, and toxins. To protect against these harmful substances, the lung has a highly developed innate host defence system that assists in rapidly clearing or detoxifying inhaled particles and pathogens; the pulmonary surfactant system is an important component of this system (LeVine and Whitsett, 2001; Zaas and Schwartz, 2005; Zhang *et al*., 2000).

Pulmonary surfactant participates in the pulmonary defence system through various roles including: (i) providing a physical barrier between the atmosphere and pulmonary circulation, (ii) directly interacting with inhaled particles deposited in the distal lung, and (iii) through interactions with other components of the pulmonary host defence system such as alveolar macrophages. Surfactant also contributes to host defence through regulating mucociliary clearance, optimizing fluid homeostasis across the alveolar-capillary barrier, directly participating in the destruction of foreign pathogens present within the airspace, and participating in the regulation of acquired host defence systems. While these properties all function to maintain normal pulmonary health throughout life, and in particular the collectin proteins,

alterations in surfactant may also represent important contributors to the pathophysiology of various lung diseases.

Role of hydrophobic surfactant components in host Defence

Currently, the main role by which the surfactant lipids, SP-B, and SP-C contribute to the pulmonary defence system is felt to be via indirect functions of their biophysical properties in forming the surface film as described above. This surface film provides a physical barrier for inhaled particles and/or pathogens and facilitates removal of these particles through mucociliary transport. Although it is difficult to attribute a relative importance to the different aspects of the complex host defence system, it is likely that this particular mechanism is responsible for the clearance of a large percentage of inhaled particles from the alveolar spaces.

Beyond the contribution of the surface film, other host defence functions of the hydrophobic surfactant components are less well established, with the majority of the evidence stemming from in vitro experiments. For example, high concentrations of an SP-B-like peptide have been shown to kill bacteria in vitro (Kaser and Skouteris, 1997) and SP-C has been shown to bind LPS (Ryan *et al*., 2006) (Augusto *et al*., 2001). The biological significance of these observations requires further investigation, although recent studies demonstrated that SP-C deficient mice were highly susceptible to *Pseudomonas aeruginosa* pneumonia (Glasser *et al*., 2005). The specific mechanisms involved in rendering these mice susceptible to bacterial infection are currently unknown.

Role of SP-A and SP-D in host Defence

The two pulmonary collectins, surfactant proteins A and D, are the pattern-recognition molecules of the pulmonary innate immune system (Crouch and Wright, 2001; Whitsett, 2005). Many in vitro and in vivo studies have shown that SP-A and SP-D play critical roles in the defence against viral, fungal, and bacterial pathogens (Crouch and Wright, 2001; Whitsett, 2005). A variety of mechanisms by which these proteins provide this protection have been established. For example, collectins can bind to microbes via their carbohydrate recognition domains (CRD), which in turn enhances the aggregation, opsonization, and clearance of these organisms via alveolar macrophages (Barr *et al*., 2000; Kuan *et al*., 1994). SP-A has also been reported to directly kill bacteria by enhancing the membrane permeability of the bacterial wall (Wu *et al*., 2003). Other mechanistic pathways include the interactions of SP-A and/or SP-D with CD14 and Toll-like receptors which in turn may regulate NF-κB expression, an important initial signalling event that determines gene expression of various inflammatory mediators within alveolar macrophages and recruited polymorphonucleocytes (Antal *et al*., 1996; Arias-Diaz *et al*., 2000; Murakami *et al*., 2002; Sano *et al*., 2000; Senft *et al*., 2005).

The numerous in vitro studies demonstrating these properties are supported by animal models of SP-A and SP-D deficiency. As noted previously, SP-A knockout (-/-) mice are phenotypically normal with respect to surfactant homeostasis and respiratory function; however, they display a significant defect in host defence properties when challenged with various microbes (Korfhagen *et al*., 1998). SP-A -/- animals display delayed clearance of organisms after administration of various bacterial and viral pathogens into their lungs and

the uptake of these pathogens via alveolar macrophages was shown to be significantly reduced compared to similarly challenged wild-type animals (LeVine *et al*., 1998, 1999, 2002). SP-A also appears to prevent bacterial spread from the lungs into the systemic circulation, since bacterial dissemination to the spleen was significantly increased in SP-A -/- mice after intratracheal administration of group B *Streptococcus* as compared to wild-type animals (LeVine *et al.*, 1997). SP-A also possesses distinct anti-inflammatory functions. SP-A knockout mice exhibited increased levels of pro-inflammatory cytokines such as TNF-alpha and IL-6 in isolated lavage fluids compared to wild-type animals after microbial challenge, with mitigation of cytokine production and bacterial clearance via intratracheal administration of exogenous recombinant SP-A (Borron *et al*., 2000; LeVine *et al*., 2002).

Phenotypically, mice lacking SP-D demonstrate features similar to that of patients with pulmonary alveolar proteinosis (PAP), typified by an accumulation of foamy alveolar macrophages, and an increase in both alveolar and cellular pools of surfactant phospholipids (Botas *et al*., 1998; Wert *et al*., 2000). These studies indicate that SP-D is required for normal surfactant metabolic processes as it regulates surfactant uptake and clearance via alveolar macrophages and possibly type II cells. Furthermore, unlike SP-A -/- mice, these phenotypic abnormalities in SP-D -/- animals make interpretation of studies involving bacterial or viral challenges more difficult. Nevertheless, when challenged with *Haemophilus influenzae*, group B *Streptococcus*, or influenza A virus inoculations, alveolar macrophages of SP-D knockout mice ingest fewer microbes than normal mice (LeVine *et al*., 2001, 2004) and exhibit significantly greater levels of pro-inflammatory cytokines compared to wild-type animals. These findings are similar in general to the SP-A -/- mice. Moreover, these abnormalities can be readily corrected by exogenous replacement with SP-D, suggesting a specific role of this material in host defence despite the phenotypic alterations observed prior to the insult.

Overall, there is overwhelming evidence both in vitro and in vivo suggesting important host defence functions for the surfactant system. These properties, in addition to other components of defence such as alveolar macrophages, neutrophils and other proteins involved in the recognition, clearance and or killing of pathogens, such as defensins (Cole and Waring, 2002; Schnapp and Harris, 1998) and lysozymes (Kalfa and Brogden, 1999), protect the lung and ensure optimal lung function. Nevertheless, there are numerous examples in which the host defence system is breached and lung injury ensues. The role of pulmonary surfactant in those situations will be discussed in subsequent sections.

8.4 Normal surfactant metabolism

As can be deduced from the descriptions of the biophysical and host defence properties of surfactant, maintaining a functional surfactant system is crucial to maintaining a healthy, optimally functioning, lung. The metabolic cycle of surfactant is responsible for maintaining this functional surfactant system and includes both intracellular and extracellular aspects.

8.4.1 Intracellular surfactant metabolism: synthesis and secretion

Although other cell types have been reported to produce some surfactant components, the alveolar type II cell is predominantly responsible for the synthesis, intracellular storage,

secretion and reuptake of pulmonary surfactant (Batenburg, 1992; Haagsman and Van Golde, 1991; Van Golde *et al*., 1987). Phospholipid substrates such as choline, glucose, and fatty acids are taken up from the vasculature by the type II cell and subsequently enter the de novo pathway for phospholipid synthesis. This process itself does not produce the specific molecular species of surfactant phospholipids, as extensive intracellular remodelling occurs to produce the high amount of dipalmitoylphosphatidylcholine present in surfactant (Post *et al*., 1983; Van Golde *et al*., 1987). Bilayers of these phospholipids are then packaged into the cytosolic storage form of surfactant called lamellar bodies (LB) prior to secretion into the alveolar space (Schmitz and Muller, 1991; Weaver *et al*., 2002).

SP-B, which is also synthesized within the type II cell, is first produced as a pro-peptide (40–42 kDa) and subsequently proteolytically cleaved at both termini to produce the active 18 kDa peptide (Voorhout *et al*., 1992; Weaver, 1998). SP-C is synthesized as a 21-kDa pro-peptide and similarly proteolytically cleaved to a 4.2-kDa monomer (Beers and Fisher, 1992; Weaver, 1998). Both of these hydrophobic proteins are routed through the Golgi apparatus and similar to the PLs are packaged into the LB structures.

Most of the hydrophilic proteins SP-A and SP-D are synthesized within the type II cell and undergo substantial post-translational modifications, including glycosylation, within the endoplasmic reticulum (McCormack, 1998). Although some reports suggest that SP-A is an initial component of LB, there also appears to be secretory pathways for this protein independent of LBs (Bakewell *et al*., 1991; Froh *et al*., 1990; Oosterlaken Dijksterhuis *et al*., 1991). As noted previously, SP-D synthesis is not unique to alveolar type II cells, although it is preferentially synthesized here and undergoes post-translational modifications prior to secretion (Crouch, 1998).

A number of different hormonal and extracellular signaling mediators (i.e. β-adrenergic agonists) may initiate surfactant secretion into the airspace (Mason and Voelker, 1998; Rooney, 2001). Within a healthy adult lung, however, physical stretch of the type II cells (such as that which occurs during normal respiration with inhalation) has been identified as an important signal for surfactant secretion (Edwards, 2001; Massaro and Massaro, 1983). To move surfactant from inside the type II cell into the airspace, exocytosis of LBs, or fusion between the apical membrane of the cell with the perimeter membrane of the LB, occurs (Chander and Wu, 1991).

8.4.2 Extracellular metabolism

Upon entering the aqueous hypophase of the airspace, LB structures unravel from the typical organized multi-lamellar storage structures to a lattice-like structure called tubular myelin (TM) and other larger organized lipid-protein structures (Sanders *et al*., 1980). Interestingly, SP-A has been shown to have lipid aggregation properties which, in part, is responsible for the formation and stability of structures such as TM (Suzuki *et al*., 1989; Voorhout *et al*., 1991). In fact, transgenic mice deficient in SP-A demonstrate a lack of TM structures present within the airspace, although these animals display normal surfactant homeostasis and respiratory function under non-stressful conditions (Korfhagen *et al*., 1998). This finding would imply that TM has a relatively minor biophysical role in the normal lung and is not essential for the formation of a surface film in this setting. Experimentally, it has been impossible to obtain pure preparations of TM. Instead researchers have utilized differential centrifugation or density gradient centrifugation techniques of lung

lavage material in order to separate subfractions of surfactant enriched in TM structures (Gross *et al*., 2000). It has been shown that two major structural forms of alveolar surfactant have been identified using these techniques; large surfactant aggregates (LA) and small surfactant aggregates (SA). LA are the heavier components and comprise the organized lipid structures, such as LB and TM, as well as the surfactant-associated proteins SP-A, SP-B, and SP-C (Putman *et al*., 1996; Veldhuizen *et al*., 1994). SA are less dense and smaller vesicular lipids that contain smaller amounts of surfactant proteins (Brackenbury *et al*., 2002; Putman *et al*., 1996).

Using these centrifugation techniques to purify surfactant subfractions, in combination with pulse-chase studies utilizing radioactive lipid precursors, it has been established that LA represent the freshly secreted forms of surfactant (Baritussio *et al*., 1984; Magoon *et al*., 1983). Moreover, isolated LA are capable of reducing surface tension to very low values when tested in vitro, and improve lung function when instilled into the lungs of surfactant-deficient animals (Brackenbury *et al*., 2002; Putz *et al*. 1994; Yamada *et al*., 1990). LA are therefore considered the biophysically active component of alveolar surfactant and are believed to be the precursor of the surface film in vivo. Unfortunately, procedures currently available do not allow for the specific isolation of the surfactant surface film. Based on the pulse chase studies mentioned above, it has also been shown that during respiration LA are 'converted' into SA (Baritussio *et al*., 1984; Magoon *et al*., 1983).

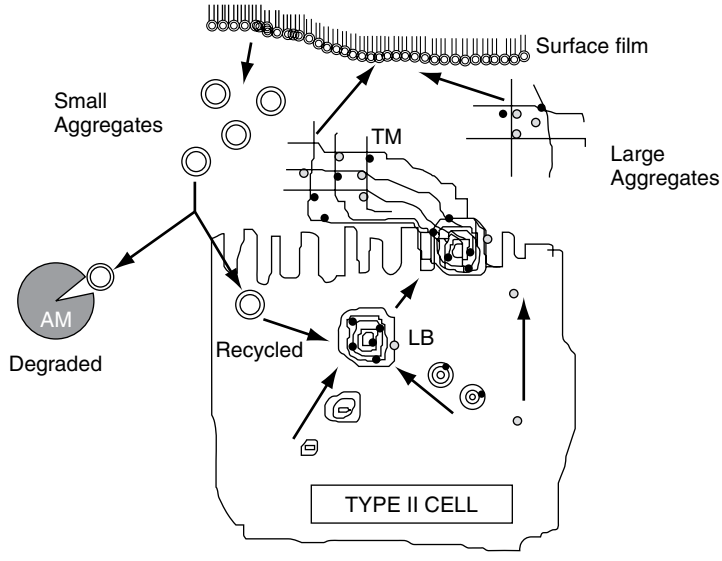


Figure 8.4 Surfactant metabolism. Within the alveolar type II cell, the concentric circles represent phospholipids and the small grey and black circles represent surfactant-associated proteins. 'LB' indicates lamellar bodies, the cytosolic storage form of surfactant. Once LBs enter the aqueous hypophase, they undergo morphological changes to form tubular myelin (TM) and adsorb to the surface film at the air–liquid interface. These components are the large aggregates and are considered the functional subfraction of surfactant. Area compression of the surface film converts LA into their non-functional form, the SA, which are cleared from the airspace by the alveolar macrophage (AM) and degraded or taken up by the type II cell for recycling

This conversion process has been studied both in vitro, using a technique called surface area cycling, and in vivo, using radioactive LA phospholipids instilled into animal lungs (Gross and Narine, 1989; Veldhuizen *et al*., 1996, 1997, 1999). Both techniques indicate that one of the critical factors for aggregate conversion within the alveolar space is a continuously changing surface area. For example, based on extensive experimental observations, it is believed that in normal lungs, LA form the surface film during inhalation, when alveolar surface area increases. Subsequently SA are formed during exhalation when the surface area is decreasing and the film is compressed. Further in vitro studies have indicated that this conversion is also mediated via the activity of a carboxylesterase molecule named convertase. The clinical relevance of convertase for surfactant metabolism has not yet been established in vivo, however (Barr *et al*., 1998; Gross, 1995; Gross and Schultz, 1990; Krishnasamy *et al*., 1997). In general, these processes result in metabolically active but relatively consistent proportion of LA and SA in normal lungs at any one time.

Following the formation of SA, these structures are then cleared from the airspace either by the alveolar macrophages or by being taken back up into the type II cell (Poelma *et al*., 2002; Rider *et al*., 1992). Alveolar macrophages are primarily responsible for the degradation of SA whereas the uptake by type II cells results in the recycling of SA phospholipids back into the formation of new LB. Figure 8.4 illustrates the overall metabolic process from synthesis of LB to the reuptake and/or degradation of the SA.

8.5 Summary: surfactant in normal lungs

As discussed above, the maintenance of a functional pulmonary surfactant system is critical for normal lung function through the reduction of surface tension at the air–liquid interface of the alveoli. Furthermore, through its host defence functions, surfactant protects the lung from inhaled particles and infectious agents. It can be deduced from these surfactant functions that alterations of surfactant may lead to a breach in pulmonary host defence, altered lung compliance, or poor gas exchange. These pathological situations are discussed below.

8.6 Surfactant in lung injury

Numerous lung injuries and diseases are known to be associated with changes to the pulmonary surfactant system, both structurally and functionally (Frerking *et al*., 2001; Griese, 1999). Some very common pulmonary diseases such as cystic fibrosis, asthma, and bronchiolitis have been shown to have alterations in surfactant isolated from these patients' lung lavage samples (Banerjee and Puniyani, 2000; Griese *et al*., 1997; Heeley *et al*., 2000; Hohlfeld *et al*., 1999). However, to what extent these alterations contribute to the pathophysiology of those diseases is largely unknown and requires further study. This section will focus on two pulmonary conditions for which there is much stronger evidence that the pulmonary surfactant system plays an active role in disease severity and progression, namely neonatal respiratory distress syndrome (NRDS) and acute respiratory distress syndrome (ARDS)

8.7 NRDS

Surfactant deficiency associated with premature delivery is the main cause of respiratory dysfunction in neonates and has been termed 'neonatal respiratory distress syndrome'. The discovery of the important role of surfactant in NRDS was made in the 1950s by Avery and Mead (Avery, 2000; Avery and Mead, 1959). They observed that infants born at less than 35 weeks' gestation had significant difficulty initiating their first breath of air (i.e. opening up the lung and establishing an air–liquid interface). Subsequent collapse of the lung with exhalation and reopening during spontaneous breathing required increasing efforts. These infants ultimately fatigued and died due to complications from hypoxemia and hypercarbia. Post-mortem examinations revealed the formation of hyaline membranes within the alveolus – indicative of extreme physical forces within the lung creating severe pulmonary edema and proteinaceous debris covering the alveolar surface. Avery was the first to establish that the repetitive alveolar collapse causing stiff and very non-compliant lungs was primarily due to high surface tension forces as a result of surfactant deficiency.

These findings resulted in major research efforts around the world focused on pulmonary surfactant administration. Several decades of work culminated in the development of exogenous surfactant therapy for these infants with NRDS (Enhorning *et al*., 1985; Robertson, 1989; Robertson and Halliday, 1998). This therapy involves the instillation of an exogenous surfactant preparation into the airway of the neonate, either immediately prior to, or shortly after, birth with immediate improvements in lung function typically observed. This therapeutic intervention has had a tremendous impact on infant mortality and is now used routinely throughout the Western world (Jacobs *et al*., 2000; Jobe and Ikegami, 2000; Kresch and Clive, 1998).

8.8 ARDS

Unlike the impressive success of surfactant therapy in NRDS, this therapeutic approach for patients with acute respiratory distress syndrome (ARDS) has had inconsistent results. This is due to the complex pathophysiology of this disorder, and the wide range of patients encompassing the clinical definition of ARDS.

In 1967, Ashbaugh first described the adult respiratory distress syndrome in 12 patients who died of respiratory failure (Ashbaugh *et al*., 1967). This condition was subsequently termed acute respiratory distress syndrome (ARDS) and is clinically characterized by hypoxemia (PaO_2/FIO_2 ratio below 200 mmHg), and decreased lung compliance with no evidence of heart failure (Bernard *et al*., 1994). ARDS represents the most severe manifestation of acute lung injury (ALI), and poses a significant burden of illness in the intensive care setting (Bellamy and Oye, 1984; Carson and Bach, 2002). The incidence of ARDS has been estimated to be 5–15 per 100 000 cases of ALI per year, if not higher, with a high mortality rate ranging from 25 to 50 per cent (Arroliga *et al*., 2002; Bersten *et al*., 2002; Goss *et al*., 2003; Villar and Slutsky, 1989).

The physiological consequences of this disease, specifically the reduced lung compliance and decreased blood oxygenation, as well as its similarity to NRDS pathologically, initially suggested that the surfactant system in these patients may be altered (Ashbaugh *et al*., 1967). This was subsequently confirmed via analysis of lung lavage material obtained from patients with ARDS (Gregory *et al*., 1991; Gunther *et al*., 1996; Hallman *et al*., 1989;

Pison *et al*., 1987; Veldhuizen *et al*., 1995). Compared to surfactant from non-ARDS patients, these patients had altered phospholipid composition, decreased amounts of surfactant-associated proteins and relatively low amounts of LA. In addition, these patients had increased amounts of serum proteins in the lavage – proteins that have been shown to inhibit surfactant function when tested both in vitro and in vivo. Together, these alterations of surfactant composition and the accumulation of serum proteins within the airspace resulted in an impaired ability of the surfactant isolated from patients with ARDS patients to reduce surface tension to low values when tested in vitro using the bubble surfactometer. These findings, together with the success of exogenous surfactant therapy in infants with NRDS, initiated investigations into the use of exogenous surfactant administration in patients with ARDS. Despite promising animal studies and a substantial number of clinical trials, exogenous surfactant therapy has yet to be shown to improve the mortality associated with this disorder (Davidson *et al*., 2006; Lewis and Veldhuizen, 2003). In the final section of this chapter, the present and future of surfactant therapy in ALI/ARDS will be discussed.

8.9 Exogenous surfactant therapy – current status and future potential

As noted, exogenous surfactant administration is now routine therapy for infants born prematurely. Although similar approaches for patients with ALI and ARDS have been somewhat disappointing to date, there is hope for the future. For example, a recent large, multi-centred, randomized clinical trial evaluated the efficacy of a recombinant SP-C-based surfactant (Venticute) in patients with severe ARDS caused by various aetiologies (Spragg *et al*., 2003). No overall improvement in mortality was observed although a post hoc analysis revealed that a subgroup of patients with 'direct' lung injuries caused by pneumonia and/or aspiration did have a significantly lower mortality when treated with surfactant compared to the control group. Moreover, these results were similar to those recently reported by Willson *et al*. in paediatric patients with direct ARDS given the natural bovine surfactant, Infasurf (Willson *et al*., 2005). Together, these results have led to an ongoing prospective clinical trial addressing the efficacy of exogenously instilled Venticute in adult patients with acute lung injury induced by direct pulmonary insults.

Based on the clinical trials to date, and the extensive preclinical data available, it is becoming evident that for exogenous surfactant to significantly impact mortality and be a cost-effective therapy in this patient population, several other factors, in addition to the aetiology of the lung injury, need to be addressed (Lewis and Veldhuizen, 1995). For example, the timing of surfactant administration has been shown to affect the outcome of animals with lung injury with earlier delivery resulting in superior outcomes (Ito *et al*., 1996; Krause and Hoehn, 2000). Although this may be a challenge clinically, there is some evidence in vivo that exogenous surfactant mitigates the progression of injury thereby rationalizing the potential of administering surfactant shortly after the onset of mechanical ventilation in patients at risk of developing ALI.

It is also possible that the specific compositional components of the administered surfactant preparation may impact outcome (Cummings *et al*., 1992). There is abundant data suggesting that SP-A may down-regulate inflammation in its role in host defence (Crouch and Wright, 2001; McCormack, 1998), however, there is no available surfactant preparation containing

this protein. Indeed, the major goal of surfactant administration previously has been to improve lung function via optimizing the biophysical properties within the airspace, and SP-A was therefore not felt to be necessary. If one of the future goals of surfactant administration is to decrease or prevent the host's inflammatory response from becoming overwhelming, more attention should be focused on manufacturing recombinant forms of this protein, and even SP-D potentially.

In summary, pulmonary surfactant is a unique substance within the lung with functional properties necessary for normal lung homeostasis. Alterations of the endogenous surfactant system have important consequences, both from a biophysical and host defence perspective. Administration of exogenous surfactant to preterm infants has significantly improved infant mortality, attesting to its biophysical relevance. While this functional role of the exogenous surfactant may also benefit patients with ALI and ARDS, it is likely that the host defence properties of a particular surfactant preparation may also be relevant in this setting. Ongoing and future research efforts should focus on which surfactant components are necessary for optimal outcomes in this patient population, as well as techniques to deliver the material in an efficient and cost-effective manner.

References

Antal, J. M., Divis, L. T., Erzurum, S. C., Wiedemann, H. P. and Thomassen, M. J. (1996) Surfactant suppresses NF-kappaB activation in human monocytic cells, *Am. J. Respir. Cell. Mol. Biol*., **14**, 374–379.

Arias-Diaz, J., Garcia-Verdugo, I., Casals, C., Sanchez-Rico, N., Vara, E. and Balibrea, J. L. (2000) Effect of surfactant protein A (SP-A) on the production of cytokines by human pulmonary macrophages, *Shock*, **14**, 300–306.

Arroliga, A. C., Ghamra, Z. W., Perez, T. A., Perez, T. P., Komara, J. J., Jr., Smith, A. and Wiedemann, H. P. (2002) Incidence of ARDS in an adult population of northeast Ohio, *Chest*, **121**, 1972–1976.

Ashbaugh, D. G., Bigelow, D. B., Petty, T. L. and Levine, B. E. (1967) Acute respiratory distress in adults, *Lancet*, **2**, 319–323.

Augusto, L., Le Blay, K., Auger, G., Blanot, D., Chaby, R. (2001) Interaction of bacterial lipopolysaccharide with mouse surfactant protein C inserted into lipid vesicles, *Am. J. Physiol. Lung Cell. Mol. Physiol*., **281**, L776–L785.

Avery, M. E. (2000) Surfactant deficiency in hyaline membrane disease: the story of discovery, *Am. J. Respir. Crit. Care Med*., **161**, 1074–1075.

Avery, M. E. and Mead, J. (1959) Surface properties in relation to atelectasis and hyaline membrane disease, *Am. J. Dis. Child*, **97**, 517–523.

Bachofen, H., Gerber, U., Gehr, P., Amrein, M. and Schurch, S. (2005) Structures of pulmonary surfactant films adsorbed to an air–liquid interface in vitro, *Biochim. Biophys. Acta*, **1720**, 59–72.

Bakewell, W. E., Viviano, C. J., Dixon, D., Smith, G. J. and Hook, G. E. (1991) Confocal laser scanning immunofluorescence microscopy of lamellar bodies and pulmonary surfactant protein A in isolated alveolar type II cells, *Lab. Invest*., **65**, 87–95.

Banerjee, R. and Puniyani, R. R. (2000) Exogenous surfactant therapy and mucus rheology in chronic obstructive airway diseases, *J. Biomater. Appl*., **14**, 243–272.

Baritussio, A., Bellina, L., Carraro, R., Rossi, A., Enzi, G., Magoon, M. W. and Mussini, I. (1984) Heterogeneity of alveolar surfactant in the rabbit: composition, morphology, and labelling of subfractions isolated by centrifugation of lung lavage, *Eur. J. Clin. Invest*., **14**, 24–29.

Barr, F., Clark, H. and Hawgood, S. (1998) Identification of a putative surfactant convertase in rat lung as a secreted serine carboxylesterase, *Am. J. Physiol*., **274** (Pt 1), L404–L410.

Barr, F. E., Pedigo, H., Johnson, T. R. and Shepherd, V. L. (2000) Surfactant protein-A enhances uptake of respiratory syncytial virus by monocytes and U937 macrophages, *Am. J. Respir. Cell. Mol. Biol*., **23**, 586–592.

Bastacky, J., Lee, C. Y., Goerke, J., Koushafar, H., Yager, D., Kenaga, L., Speed, T. P., Chen, Y. and Clements, J. A. (1995) Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung, *J. Appl. Physiol*., **79**, 1615–1628.

Batenburg, J. J. (1992) Surfactant phospholipids: synthesis and storage, *Am. J. Physiol*., **262**, L367–L385.

Beers, M. F. and Fisher, A. B. (1992) Surfactant protein C: a review of its unique properties and metabolism, *Am. J. Physiol*., **263**, L151–L160.

Bellamy, P. E. and Oye, R. K. (1984) Adult respiratory distress syndrome: hospital charges and outcome according to underlying disease, *Crit. Care Med*., **12**, 622–625.

Bernard, G. R., Artigas, A., Brigham, K. L., Carlet, J., Falke, K., Hudson, L., Lamy, M., Legall, J. R., Morris, A., Spragg, R. and the Consensus Committee. (1994) The American–European consensus conference on ARDS: definitions, mechanisms, relevant outcomes, and clinical trial coordination, *Am. J. Respir. Crit. Care Med*., **149**, 818–824.

Bersten, A. D., Edibam, C., Hunt, T. and Moran, J. (2002) Incidence and mortality of acute lung injury and the acute respiratory distress syndrome in three Australian States, *Am. J. Respir. Crit. Care Med*., **165**, 443–448.

Borron, P., McIntosh, J. C., Korfhagen, T. R., Whitsett, J. A., Taylor, J. and Wright, J. R. (2000) Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo, *Am. J. Physiol. Lung Cell. Mol. Physiol*., **278**, L840–L847.

Botas, C., Poulain, F., Akiyama, J., Brown, C., Allen, L., Goerke, J., Clements, J., Carlson, E., Gillespie, A. M., Epstein, C. and Hawgood, S. (1998) Altered surfactant homeostasis and alveolar type ii cell morphology in mice lacking surfactant protein d, *Proc. Natl Acad. Sci. U.S.A.*, **95**, 11869–11874.

Brackenbury, A. M., Malloy, J. L., McCaig, L. A., Yao, L. J., Veldhuizen, R. A. and Lewis, J. F. (2002) Evaluation of alveolar surfactant aggregates in vitro and in vivo, *Eur. Respir. J*., **19**, 41–46.

Carson, S. S. and Bach, P. B. (2002) The epidemiology and costs of chronic critical illness, *Crit. Care Clin*., **18**, 461–476.

Chander, A. and Wu, R. D. (1991) In vitro fusion of lung lamellar bodies and plasma membrane is augmented by lung synexin, *Biochim. Biophys. Acta*, **1086**, 157–166.

Cockshutt, A. M., Weitz, J. and Possmayer, F. (1990) Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro, *Biochemistry*, **29**, 8424–8429.

Cole, A. M. and Waring, A. J. (2002) The role of defensins in lung biology and therapy, *Am. J. Respir. Med*., **1**, 249–259.

Crouch, E. C. (1998) Structure, biologic properties, and expression of surfactant protein D (SP-D), *Biochim. Biophys. Acta*, **1408**, 278–289.

Crouch, E. and Wright, J. (2001) Surfactant proteins a and d and pulmonary host defense, *Annu. Rev. Physiol*., **63**, 521–554.

Crouch, E., Hartshorn, K. and Ofek, I. (2000) Collectins and pulmonary innate immunity, *Immunol. Rev*., **173**, 52–65.

Cummings, J. J., Holm, B. A., Hudak, M. L., Hudak, B. B., Ferguson, W. H. and Egan, E. A. (1992) A controlled clinical comparison of four different surfactant preparations in surfactant-deficient preterm lambs, *Am. Rev. Respir. Dis*., **145**, 999–1004.

Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Lowenadler, B. and Jornvall, H. (1990) Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP- B lacks covalently linked fatty acyl groups, *Proc. Natl Acad. Sci. U.S.A*., **87**, 2985–2989.

Davidson, W. J., Dorscheid, D., Spragg, R., Schulzer, M., Mak, E. and Ayas, N. T. (2006) Exogenous pulmonary surfactant for the treatment of adult patients with acute respiratory distress syndrome: results of a meta-analysis, *Crit. Care*, **10**, R41.

Edwards, Y. S. (2001) Stretch stimulation: its effects on alveolar type II cell function in the lung, *Comp. Biochem. Physiol. A Mol. Integr. Physiol*., **129**, 245–260.

Enhorning, G. (1977) Pulsating bubble technique for evaluating pulmonary surfactant, *J. Appl. Physiol*., **43**, 198–203.

Enhorning, G., Shennan, A., Possmayer, F., Dunn, M., Chen, C. P. and Milligan, J. (1985) Prevention of neonatal respiratory distress syndrome by tracheal instillation of surfactant: a randomized clinical trial, *Pediatrics*, **76**, 145–153.

Frerking, I., Gunther, A., Seeger, W. and Pison, U. (2001) Pulmonary surfactant: functions, abnormalities and therapeutic options, *Intensive Care Med*., **27**, 1699–1717.

Froh, D., Ballard, P. L., Williams, M. C., Gonzales, J., Goerke, J., Odom, M. W. and Gonzales, L. W. (1990) Lamellar bodies of cultured human fetal lung: content of surfactant protein A (SP-A), surface film formation and structural transformation in vitro, *Biochim. Biophys. Acta*, **1052**, 78–89.

Glasser, S. W., Burhans, M. S., Korfhagen, T. R., Na, C. L., Sly, P. D., Ross, G. F., Ikegami, M. and Whitsett, J. A. (2001) Altered stability of pulmonary surfactant in SP-C-deficient mice, *Proc. Natl Acad. Sci. U.S.A*., **98**, 6366–6371.

Glasser, S. W., Detmer, E. A., Ikegami, M., Na, C. L., Stahlman, M. T. and Whitsett, J. A. (2003) Pneumonitis and emphysema in sp-C gene targeted mice, *J. Biol. Chem*., **278**, 14291–14298.

Glasser, S.W., Maxfield, M.D., Richardson, T.T., Whitsett, J.A., Baatz, J.E., Wert, S.E., Senft, A.P., Korfhagen, T.R., (2005) SP-C deficient mice are susceptible to *P. aeruginosa* infection, *Proc. Am. Thorac. Soc*., **2**, A454.

Goerke, J. (1998) Pulmonary surfactant: functions and molecular composition, *Biochim. Biophys. Acta*, **1408**, 79–89.

Goss, C. H., Brower, R. G., Hudson, L. D. and Rubenfeld, G. D. (2003) Incidence of acute lung injury in the United States, *Crit. Care Med*., **31**, 1607–1611.

Gregory, T. J., Longmore, W. J., Moxley, M. A., Whitsett, J. A., Reed, C. R., Fowler, A. A., Maunder, R. J., Crim, C. and Hyers, T. M. (1991) Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome, *J. Clin. Invest*., **88**, 1976–1981.

Griese, M. (1999) Pulmonary surfactant in health and human lung diseases: state of the art, *Eur. Respir. J*., **13**, 1455–1476.

Griese, M., Birrer, P. and Demirsoy, A. (1997) Pulmonary surfactant in cystic fibrosis, *Eur. Respir. J*., **10**, 1983–1988.

Gross, N. J. (1995) Extracellular metabolism of pulmonary surfactant: the role of a new serine protease, *Annu. Rev. Physiol*., **57**, 135–150.

Gross, N. J. and Narine, K. R. (1989) Surfactant subtypes of mice: metabolic relationships and conversion in vitro, *J. Appl. Physiol*., **67**, 414–421.

Gross, N. J. and Schultz, R. M. (1990) Serine proteinase requirement for the extra-cellular metabolism of pulmonary surfactant, *Biochim. Biophys. Acta*, **1044**, 222–230.

Gross, N. J., Kellam, M., Young, J., Krishnasamy, S. and Dhand, R. (2000) Separation of alveolar surfactant into subtypes. A comparison of methods, *Am. J. Respir. Crit. Care Med*., **162**, 617–622.

Gunther, A., Siebert, C., Schmidt, R., Ziegler, S., Grimminger, F., Yabut, M., Temmesfeld, B., Walmrath, D., Morr, H. and Seeger, W. (1996) Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema, *Am. J. Respir. Crit. Care Med*., **153**, 176–184.

Haagsman, H. P. and Diemel, R. V. (2001) Surfactant-associated proteins: functions and structural variation, *Comp. Biochem. Physiol. A Mol. Integr. Physiol*., **129**, 91–108.

Haagsman, H. P. and Van Golde, L. M. (1991) Synthesis and assembly of lung surfactant, *Annu. Rev. Physiol*., **53**, 441–464.

Haagsman, H. P., Hawgood, S., Sargeant, T., Buckley, D., White, R. T., Drickamer, K. and Benson, B. J. (1987) The major lung surfactant protein, SP 28–36, is a calcium-dependent, carbohydrate-binding protein, *J. Biol. Chem*., **262**, 13877–13880.

Hallman, M., Maasilta, P., Sipila, I. and Tahvanainen, J. (1989) Composition and function of pulmonary surfactant in adult respiratory distress syndrome, *Eur. Respir. J. Suppl*., **3**, 104s–108s.

Hawgood, S. and Shiffer, K. (1991) Structures and properties of the surfactant-associated proteins, *Annu. Rev. Physiol*., **53**, 375–394.

Hawgood, S., Derrick, M. and Poulain, F. (1998) Structure and properties of surfactant protein B, *Biochim. Biophys. Acta*, **1408**, 150–160.

Heeley, E. L., Hohlfeld, J. M., Krug, N. and Postle, A. D. (2000) Phospholipid molecular species of bronchoalveolar lavage fluid after local allergen challenge in asthma, *Am. J. Physiol. Lung Cell. Mol. Physiol*., **278**, L305–L311.

Hohlfeld, J. M., Ahlf, K., Enhorning, G., Balke, K., Erpenbeck, V. J., Petschallies, J., Hoymann, H. G., Fabel, H. and Krug, N. (1999) Dysfunction of pulmonary surfactant in asthmatics after segmental allergen challenge, *Am. J. Resp. Crit. Care Med*., **159**, 1803–1809.

Ito, Y., Goffin, J., Veldhuizen, R., Joseph, M., Bjarneson, D., McCaig, L., Yao, L.-J., Marcou, J. and Lewis, J. (1996) Timing of exogenous surfactant administration in a rabbit model of acute lung injury, *J. Appl. Physiol*., **80**, 1357–1364.

Jacobs, S. E., O'Brien, K., Inwood, S., Kelly, E. N. and Whyte, H. E. (2000) Outcome of infants 23–26 weeks' gestation pre and post surfactant, *Acta Paediatr*., **89**, 959–965.

Jobe, A. H. and Ikegami, M. (2000) Lung development and function in preterm infants in the surfactant treatment era, *Annu. Rev. Physiol*., **62**, 825–846.

Johansson, J. (1998) Structure and properties of surfactant protein C, *Biochim. Biophys. Acta*, **1408**, 161–172.

Kalfa, V. C. and Brogden, K. A. (1999) Anionic antimicrobial peptide–lysozyme interactions in innate pulmonary immunity, *Int. J. Antimicrob. Agents*, **13**, 47–51.

Kaser, M.R. and Skouteris, G.G. (1997) Inhibition of bacterial growth by synthetic SP-B1-78 peptides, *Peptides*, **18**, 1441–1444.

Korfhagen, T. R., LeVine, A. M. and Whitsett, J. A. (1998) Surfactant protein A (SP-A) gene targeted mice, *Biochim. Biophys. Acta*, **1408**, 296–302.

Krause, M. F. and Hoehn, T. (2000) Timing of surfactant administration determines its physiologic response in a rabbit model of airway lavage, *Biol. Neonate*, **77**, 196–202.

Kresch, M. J. and Clive, J. M. (1998) Meta-analyses of surfactant replacement therapy of infants with birth weights less than 2000 grams, *J. Perinatol*., **18**, 276–283.

Krishnasamy, S., Gross, N. J., Teng, A. L., Schultz, R. M. and Dhand, R. (1997) Lung 'surfactant convertase' is a member of the carboxylesterase family, *Biochem. Biophys. Res. Commun*., **235**, 180–184.

Kuan, S.-F., Persson, A. and Crouch, E. (1994) Lectin-mediated interaction of surfactant protein D with alveolar macrophages, *Am. J. Respir. Cell. Mol. Biol*., **10**, 430–436.

Kuroki, Y. and Sano, H. (1999) Functional roles and structural analysis of lung collectins SP-A and SP- D, *Biol. Neonate*, **76**, (Suppl 1), 19–21.

LeVine, A. M. and Whitsett, J. A. (2001) Pulmonary collectins and innate host defense of the lung, *Microbes Infect*., **3**, 161–166.

LeVine, A. M., Bruno, M. D., Huelsman, K. M., Ross, G. F., Whitsett, J. A. and Korfhagen, T. R. (1997) Surfactant protein A-deficient mice are susceptible to group B streptococcal infection, *J. Immunol*., **158**, 4336–4340.

LeVine, A. M., Kurak, K. E., Bruno, M. D., Stark, J. M., Whitsett, J. A. and Korfhagen, T. R. (1998) Surfactant protein-A-deficient mice are susceptible to *Pseudomonas aeruginosa* infection, *Am. J. Respir. Cell. Mol. Biol*., **19**, 700–708.

LeVine, A. M., Gwozdz, J., Stark, J., Bruno, M., Whitsett, J. and Korfhagen, T. (1999) Surfactant protein-A enhances respiratory syncytial virus clearance in vivo, *J. Clin. Invest*., **103**, 1015–1021.

LeVine, A. M., Whitsett, J. A., Hartshorn, K. L., Crouch, E. C. and Korfhagen, T. R. (2001) Surfactant protein D enhances clearance of influenza A virus from the lung in vivo, *J. Immunol*., **167**, 5868–5873.

LeVine, A. M., Hartshorn, K., Elliott, J., Whitsett, J. and Korfhagen, T. (2002) Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection, *Am. J. Physiol. Lung Cell. Mol. Physiol*., **282**, L563–L572.

LeVine, A. M., Elliott, J., Whitsett, J. A., Srikiatkhachorn, A., Crouch, E., DeSilva, N. and Korfhagen, T. (2004) Surfactant protein-d enhances phagocytosis and pulmonary clearance of respiratory syncytial virus, *Am. J. Respir. Cell. Mol. Biol*., **31**, 193–199.

Lewis, J. F. and Veldhuizen, R. A. W. (1995) Factors influencing efficacy of exogenous surfactant in acute lung injury, *Biol. Neonate*, **67** (Suppl 1), 48–60.

Lewis, J. F. and Veldhuizen, R. (2003) The role of exogenous surfactant in the treatment of acute lung injury, *Annu. Rev. Physiol*, **65**, 613–642.

Magoon, M. W., Wright, J. R., Baritussio, A., Williams, M. C., Goerke, J., Benson, B. J., Hamilton, R. L. and Clements, J. A. (1983) Subfractionation of lung surfactant. Implications for metabolism and surface activity, *Biochim. Biophys. Acta*, **750**, 18–31.

Mason, R. J. and Voelker, D. R. (1998) Regulatory mechanisms of surfactant secretion, *Biochim. Biophys. Acta*, **1408**, 226–240.

Massaro, G. D. and Massaro, D. (1983) Morphologic evidence that large inflations of the lung stimulate secretion of surfactant, *Am. Rev. Respir Dis*., **127**, 235–236.

McCormack, F. X. (1998) Structure, processing and properties of surfactant protein A, *Biochim. Biophys. Acta*, **1408**, 109–131.

McCormack, F. X. and Whitsett, J. A. (2002) The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung, *J. Clin. Invest*., **109**, 707–712.

Murakami, S., Iwaki, D., Mitsuzawa, H., Sano, H., Takahashi, H., Voelker, D. R., Akino, T. and Kuroki, Y. (2002) Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-alpha secretion in U937 cells and alveolar macrophages by direct interaction with toll-like receptor 2, *J. Biol. Chem*., **277**, 6830–6837.

Oosterlaken Dijksterhuis, M. A., van Eijk, M., van Buel, B. L., Van Golde, L. M. and Haagsman, H. P. (1991) Surfactant protein composition of lamellar bodies isolated from rat lung, *Biochem. J*., **274**, 115–119.

Persson, A., Chang, D., Rust, K., Moxley, M., Longmore, W. and Crouch, E. (1989) Purification and biochemical characterization of CP4 (SP-D), a collagenous surfactant-associated protein, *Biochemistry*, **28**, 6361–6367.

Piknova, B., Schief, W. R., Vogel, V., Discher, B. M. and Hall, S. B. (2001) Discrepancy between phase behavior of lung surfactant phospholipids and the classical model of surfactant function, *Biophys. J*., **81**, 2172–2180.

Piknova, B., Schram, V. and Hall, S. B. (2002) Pulmonary surfactant: phase behavior and function, *Curr. Opin. Struct. Biol*., **12**, 487–494.

Pison, U., Gono, E., Joka, T. and Obertacke, U. (1987) Phospholipid lung profile in adult respiratory distress syndrome – evidence for surfactant abnormality, *Prog. Clin. Biol. Res*., **236A**, 517–523.

Poelma, D. L., Zimmermann, L. J., Scholten, H. H., Lachmann, B. and Van Iwaarden, J. F. (2002) In vivo and in vitro uptake of surfactant lipids by alveolar type II cells and macrophages, *Am. J. Physiol. Lung Cell. Mol. Physiol*., **283**, L648–L654.

Possmayer, F. (1988) A proposed nomenclature for pulmonary surfactant-associated proteins, *Am. Rev. Respir. Dis*., **138**, 990–998.

Post, M., Schuurmans, E. A., Batenburg, J. J. and Van Golde, L. M. (1983) Mechanisms involved in the synthesis of disaturated phosphatidylcholine by alveolar type II cells isolated from adult rat lung, *Biochim. Biophys. Acta*, **750**, 68–77.

Postle, A. D., Heeley, E. L. and Wilton, D. C. (2001) A comparison of the molecular species compositions of mammalian lung surfactant phospholipids, *Comp. Biochem. Physiol. A. Mol. Integr. Physiol*., **129**, 65–73.

Putman, E., Creuwels, L. A., Van Golde, L. M. and Haagsman, H. P. (1996) Surface properties, morphology and protein composition of pulmonary surfactant subtypes, *Biochem. J*., **320** (Pt 2), 599–605.

Putz, G., Goerke, J. and Clements, J. A. (1994) Surface activity of rabbit pulmonary surfactant subfractions at different concentrations in a captive bubble, *J. Appl. Physiol*., **77**, 597–605.

Qanbar, R. and Possmayer, F. (1995) On the surface activity of surfactant-associated protein C (SP-C): effects of palmitoylation and pH, *Biochim. Biophys. Acta*, **1255**, 251–259.

Rider, E. D., Ikegami, M. and Jobe, A. H. (1992) Localization of alveolar surfactant clearance in rabbit lung cells, *Am. J. Physiol*., **263**, L201–L209.

Robertson, B. (1989) Background to neonatal respiratory distress syndrome and treatment with exogenous surfactant, *Dev. Pharmacol. Ther*., **13**, 159–163.

Robertson, B. and Halliday, H. L. (1998) Principles of surfactant replacement, *Biochim. Biophys. Acta*, **1408**, 346–361.

Rodriguez-Capote, K., McCormack, F. X. and Possmayer, F. (2003) Pulmonary surfactant protein-A (SP-A) restores the surface properties of surfactant after oxidation by a mechanism that requires the Cys6 interchain disulfide bond and the phospholipid binding domain, *J. Biol. Chem*., **278**, 20461–20474.

Rooney, S. A. (2001) Regulation of surfactant secretion, *Comp. Biochem. Physiol. A Mol. Integr. Physiol*., **129**, 233–243.

Ryan, M. A., Akinbi, H. T., Serrano, A. G., Perez-Gil, J., Wu, H., McCormack, F. X. and Weaver, T. E. (2006) Antimicrobial activity of native and synthetic surfactant protein B peptides, *J. Immunol*., **176**, 416–425.

Sanders, R. L., Hassett, R. J. and Vatter, A. E. (1980) Isolation of lung lamellar bodies and their conversion to tubular myelin figures in vitro, *Anat. Rec*., **198**, 485–501.

Sano, H., Chiba, H., Iwaki, D., Sohma, H., Voelker, D. R. and Kuroki, Y. (2000) Surfactant proteins A and D bind CD14 by different mechanisms, *J. Biol. Chem*., **275**, 22442–22451.

Schmitz, G. and Muller, G. (1991) Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids, *J. Lipid. Res*., **32**, 1539–1570.

Schnapp, D. and Harris, A. (1998) Antibacterial peptides in bronchoalveolar lavage fluid, *Am. J. Respir. Cell. Mol. Biol*., **19**, 352–356.

Schurch, S., Bachofen, H., Goerke, J. and Possmayer, F. (1989) A captive bubble method reproduces the in situ behavior of lung surfactant monolayers, *J. Appl. Physiol*., **67**, 2389–2396.

Schurch, S., Qanbar, R., Bachofen, H. and Possmayer, F. (1995) The surface-associated surfactant reservoir in the alveolar lining, *Biol. Neonate*, **67** (Suppl 1), 61–76.

Senft, A. P., Korfhagen, T. R., Whitsett, J. A., Shapiro, S. D. and LeVine, A. M. (2005) Surfactant protein-D regulates soluble CD14 through matrix metalloproteinase-12, *J. Immunol*., **174**, 4953–4959.

Shelley, S. A., Paciga, J. E. and Balis, J. U. (1984) Lung surfactant phospholipids in different animal species, *Lipids*., **19**, 857–862.

Spragg, R. G., Lewis, J. F., Wurst, W., Hafner, D., Baughman, R. P., Wewers, M. D. and Marsh, J. J. (2003) Treatment of acute respiratory distress syndrome with recombinant surfactant protein C surfactant, *Am. J. Respir. Crit. Care Med*., **167**, 1562–1566.

Strayer, D. S., Herting, E., Sun, B. and Robertson, B. (1996) Antibody to surfactant protein A increases sensitivity of pulmonary surfactant to inactivation by fibrinogen in vivo, *Am. J. Respir. Crit. Care Med*., **153**, 1116–1122.

Suzuki, Y., Fujita, Y. and Kogishi, K. (1989) Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant, *Am. Rev. Respir. Dis*., **140**, 75–81.

Tokieda, K., Whitsett, J. A., Clark, J. C., Weaver, T. E., Ikeda, K., McConnell, K. B., Jobe, A. H., Ikegami, M. and Iwamoto, H. S. (1997) Pulmonary dysfunction in neonatal SP-B-deficient mice, *Am. J. Physiol*., **273**, L875–L882.

Van Golde, L. M., de Vries, A. C. and Batenburg, J. J. (1987) Aspects of metabolism and storage of pulmonary surfactant: experiments with isolated type II pneumocytes and lamellar bodies, *Eur. J. Respir. Dis. Suppl*., **153**, 182–188.

Veldhuizen, E. J. and Haagsman, H. P. (2000) Role of pulmonary surfactant components in surface film formation and dynamics, *Biochim. Biophys. Acta*, **1467**, 255–270.

Veldhuizen, R. and Possmayer, F. (2004) Phospholipid metabolism in lung surfactant, *Subcell. Biochem*., **37**, 359–388.

Veldhuizen, R. A. W., Hearn, S. A., Lewis, J. F. and Possmayer, F. (1994) Surface-area cycling of different surfactant preparations: SP-A and SP-B are essential for large-aggregate integrity, *Biochem. J*., **300**, 519–524.

Veldhuizen, R. A., McCaig, L. A., Akino, T. and Lewis, J. F. (1995) Pulmonary surfactant subfractions in patients with the acute respiratory distress syndrome, *Am. J. Respir. Crit. Care Med*., **152**, 1867–1871.

Veldhuizen, R. A. W., Marcou, J., Yao, L.-J., McCaig, L., Ito, Y. and Lewis, J. F. (1996) Alveolar surfactant aggregate conversion in ventilated normal and injured rabbits, *Am. J. Physiol*., **270**, L152–L158.

Veldhuizen, R. A. W., Ito, Y., Marcou, J., Yao, L. J., McCaig, L. and Lewis, J. F. (1997) Effects of lung injury on pulmonary surfactant aggregate conversion in vivo and in vitro, *Am. J. Physiol*., **16**, L872–L878.

Veldhuizen, R. A., Yao, L. and Lewis, J. F. (1999) An examination of the different variables affecting surfactant aggregate conversion in vitro, *Exp. Lung. Res*., **25**, 127–141.

Veldhuizen, E. J., Batenburg, J. J., Van Golde, L. M. and Haagsman, H. P. (2000) The role of surfactant proteins in DPPC enrichment of surface films, *Biophys. J*., **79**, 3164–3171.

Villar, J. and Slutsky, A. S. (1989) The incidence of the adult respiratory distress syndrome, *Am. Rev. Respir. Dis*., **140**, 814–816.

Voorhout, W. F., Veenendaal, T., Haagsman, H. P., Verkleij, A. J., Van Golde, L. M. and Geuze, H. J. (1991) Surfactant protein A is localized at the corners of the pulmonary tubular myelin lattice, *J. Histochem. Cytochem*., **39**, 1331–1336.

Voorhout, W. F., Veenendaal, T., Haagsman, H. P., Weaver, T. E., Whitsett, J. A. and Geuze, H. J. (1992) Intracellular processing of pulmonary surfactant protein B in an endosomal/lysosomal compartment, *Am. J. Physiol*., **263**, L479–L486.

Weaver, T. E. (1998) Synthesis, processing and secretion of surfactant proteins B and C, *Biochim. Biophys. Acta*, **1408**, 173–179.

Weaver, T. E. and Beck, D. C. (1999) Use of knockout mice to study surfactant protein structure and function, *Biol. Neonate*, **76** (Suppl 1), 15–18.

Weaver, T. E., Na, C. L. and Stahlman, M. (2002) Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant, *Semin. Cell. Dev. Biol*., **13**, 263–270.

Wert, S. E., Yoshida, M., LeVine, A. M., Ikegami, M., Jones, T., Ross, G. F., Fisher, J. H., Korfhagen, T. R. and Whitsett, J. A. (2000) Increased metalloproteinase activity, oxidant production, and emphysema in surfactant protein D gene-inactivated mice, *Proc. Natl Acad. Sci. U.S.A*., **97**, 5972–5977.

Whitsett, J. A. (2005) Surfactant proteins in innate host defense of the lung, *Biol. Neonate*, **88**, 175–180.

Willson, D. F., Thomas, N. J., Markovitz, B. P., Bauman, L. A., DiCarlo, J. V., Pon, S., Jacobs, B. R., Jefferson, L. S., Conaway, M. R. and Egan, E. A. (2005) Effect of exogenous surfactant (calfactant) in pediatric acute lung injury: a randomized controlled trial, *JAMA*, **293**, 470–476.

Wu, H., Kuzmenko, A., Wan, S., Schaffer, L., Weiss, A., Fisher, J. H., Kim, K. S. and McCormack, F. X. (2003) Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability, *J. Clin. Invest*., **111**, 1589–1602.

Yamada, T., Ikegami, M. and Jobe, A. H. (1990) Effects of surfactant subfractions on preterm rabbit lung function, *Pediatr. Res*., **27**, 592–598.

Yu, S. H. and Possmayer, F. (1990) Role of bovine pulmonary surfactant-associated proteins in the surface-active property of phospholipid mixtures, *Biochim. Biophys. Acta*, **1046**, 233–241.

Yu, S. H. and Possmayer, F. (1992) Effect of pulmonary surfactant protein B (SP-B) and calcium on phospholipid adsorptionand squeeze-out of phosphatidylglycerol from binary phospholipid monolayers containing dipalmitoylphosphatidylcholine, *Biochim. Biophys. Acta*, **1126**, 26–34.

Yu, S. H., Chung, W., Olafson, R. W., Harding, P. G. and Possmayer, F. (1987) Characterization of the small hydrophobic proteins associated with pulmonary surfactant, *Biochim. Biophys. Acta*, **921**, 437–448.

Zaas, A. K. and Schwartz, D. A. (2005) Innate immunity and the lung: defense at the interface between host and environment, *Trends. Cardiovasc. Med*., **15**, 195–202.

Zhang, P., Summer, W. R., Bagby, G. J. and Nelson, S. (2000) Innate immunity and pulmonary host defense, *Immunol. Rev*., **173**, 39–51.

9

Microbial Recognition by Epithelium

Catherine M. Greene[1] and **Stephen G. J. Smith**[2]
[1]*Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin, Ireland*
[2]*Department of Clinical Microbiology, Trinity College, Trinity Centre for Health Sciences, St. James's Hospital, Dublin, Ireland*

9.1 Introduction

The pulmonary epithelium is a key component of the host's innate immune defences against microbial pathogens. Airway epithelial cells are equipped with an array of factors that collectively act as a sentinel system that can rapidly and effectively detect and respond to microbial insults. The ability to sense pathogens is the principal property of pattern recognition receptors (PRRs). Epithelial cells within the lung utilize a variety of PRRs to maximize their capacity to recognize, respond to and ultimately control pulmonary invasion by microbes. There is a growing repertoire of functional PRRs, which is now known to include both transmembrane and cytosolic receptors belonging to a number of distinct families. The Toll-like receptor (TLR) family represents the most significant component of pulmonary PRRs and this dynamic family of transmembrane proteins can recognize and discriminate a diverse array of microbial antigens. A number of non-TLR transmembrane receptors such as complement receptors also are adjunct in pulmonary innate immune responses and the recently emergent cytosolic PRRs also fulfil an essential role in effective microbial recognition by epithelium.

9.2 TLRs – identification and structure

The TLR family constitutes an important unit of the innate immune system and although most commonly considered to be associated with immune cell responses, TLRs are also known to be functionally expressed on a variety of other cell types including airway epithelial

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

cells. These cells represent a large percentage of the cellular content of the airway, provide a barrier to infection and an active defence mechanism against invading microbes. Their contribution to the inflammatory response in the lung is significant.

TLRs were first identified in the fruitfly *Drosophila melanogaster*. *Drosophila* or dToll was initially characterized as a protein with an important role in regulating embryogenesis and was subsequently found to act as a key receptor regulating antimicrobial defence in the adult fly (Lemaitre *et al*., 1996). The seminal discovery of structural and functional similarities between dToll and the mammalian Type I interleukin-1 receptor (IL-1RI) (Gay and Keith, 1991), an important receptor in innate immunity, led to a flurry of research in this area, culminating in the identification and partial characterization of 10 human TLRs, each having sequence similarity to the cytosolic signalling domain of IL-1RI. TLRs are germ-line encoded pattern recognition receptors, and each is implicated in the innate immune response (Akira and Hemmi, 2003). Their expression is widespread and includes, but is not limited to, cells of myeloid and lymphoid origin, endothelial and epithelial cells.

TLRs are type I transmembrane receptors, each with an extracellular leucine-rich repeat (LRR) ligand recognition domain and an intracellular signalling domain integrating the functional signature motif of TLRs, a TIR (Toll/interleukin-1 receptor) domain. The cytosolic TIR domain is a conserved region of approximately 200 amino acids (O'Neill and Greene, 1998; O'Neill, 2002) essential for signalling, while the external LRR motifs are thought to be the regions that confer specificity to TLRs with respect to their pattern recognition properties, and may also be involved in TLR dimerization (Bell *et al*., 2003). TLR4 was the first mammalian TLR to be identified and is the best characterized to date. Its identity as the mammalian LPS receptor initially came from studies on the LPS hypo-responsive mouse strain C3H/HeJ (Poltorak *et al*., 1998). These mice have a dominant-negative Pro712His mutation in the TIR domain of their TLR4 and consequently can withstand challenge with lethal doses of LPS.

9.3 TLR agonist recognition and specificity

The principal role of TLRs is to facilitate the recognition and discrimination of invading microbes, and to induce an appropriate immune response. TLRs become activated by specific agonists derived from diverse species including bacteria, viruses, mycoplasma, yeasts and protozoa (Figure 9.1). TLR4 is the principal receptor for bacterial lipopolysaccharide (LPS) and, for example, on airway epithelial cells it is known to contribute to the recognition of *Haemophilus influenza* (Wang *et al*., 2002). TLR4 can also recognize other microbial agonists including the pneumococcal virulence factor pneumolysin (Malley *et al*., 2003), Hsp60 from *Chlamydia pneumoniae*, flavolipin, murine retroviruses and fusion protein from respiratory syncytial virus (RSV) (Gomi *et al*., 2002; Kurt-Jones *et al*., 2000; Rassa *et al*., 2002; Sasu *et al*., 2001).

Of all the TLRs, TLR2 recognizes the broadest repertoire of agonists and is a functionally important PRR expressed by the bronchial epithelium, which can respond to lipoteichoic acid, peptidoglycan and *Mycoplasma pneumoniae* (Armstrong *et al*., 2004; Chu *et al*., 2005; Gon *et al*., 2004). TLR2 can heterdimerize with other TLRs to confer responsiveness to a diverse array of agonists (Wetzler, 2003). In conjunction with TLR1, it recognizes triacylated lipopeptides, Gram-positive lipoteichoic acid and *Streptococcus pneumoniae* (Schmeck *et al*.,

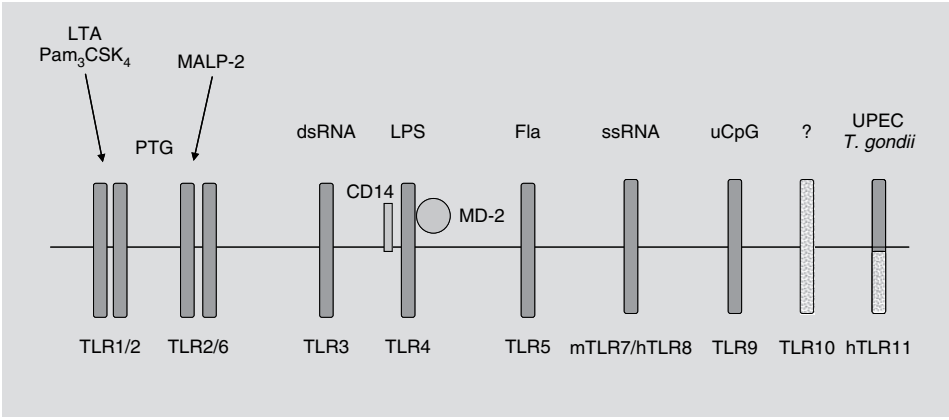


Figure 9.1 Principal microbial TLR agonists. LTA, lipoteichoic acid; Pam_3CSK_4, triacylated lipopeptide; PTG, peptidoglycan; MALP-2, diacylated lipopeptide; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; Fla, flagellin; ssRNA, single-stranded RNA; uCpG, unmethylated CpG dinucleotide motifs; UPEC, uropathogenic *Escherichia coli*; hTLR, human TLR; mTLR, murine TLR. TLR10 is an orphan receptor; hTLR11 is depicted as truncated and non-functional

2006) amongst others, whereas with TLR6, it can respond to diacylated lipopeptides such as MALP-2 from mycoplasma.

TLR3 has a role in recognition of viral nucleic acids, specifically double-stranded (ds)RNA (Alexopoulou *et al*., 2001; Hewson *et al*., 2005; Ritter *et al*., 2005) a by-product of viral replication in infected cells. TLR3 has been shown to participate in lung epithelial cell recognition of rhinovirus (Hewson *et al*., 2005) and influenza A virus (Guillot *et al*., 2004a) and contributes to innate immune responses induced by RSV (Rudd *et al*., 2006). Flagellin is the protein monomer of bacterial flagellae expressed on Gram-negative bacteria. Flagellin induces TLR5-dependent signalling (Hayashi *et al*., 2001) on lung epithelial cells in response to *Pseudomonas aeruginosa, Legionella pneumophila* and *Bordetella bronchispetica* (Hawn *et al*., 2003; Lopez-Boado *et al*., 2005; Sadikot *et al*., 2005; Zhang *et al*., 2005). Interestingly TLR4 has been implicated in some TLR5 responses (Honko and Mizel, 2005) whilst TLR2 has a role in recognition of *Pseudomonas* flagellin by TLR5 (Adamo *et al*., 2004). This is not altogether unexpected given TLR2's known ability to heterodimerize with other TLRs and respond to multiple agonists.

Although expression of TLRs 7 and 8 on lung epithelium has not been well characterized to date, these receptors are known to have a role in the anti-viral response (Hemmi *et al*., 2002; Jurk *et al*., 2002). Guanosine- and uridine-rich single-stranded (ss)RNA found in many viruses, represent the major agonists for murine TLR7 and human TLR8 (Diebold *et al*., 2004; Heil *et al*., 2004). Microbial DNA characterized by unmethylated CpG (uCpG) dinucleotide motifs activate TLR9 (Hemmi *et al*., 2000). These occur frequently in bacterial but not mammalian DNA. Human TLR10 is an orphan member of the TLR family (Hasan *et al*., 2005). It has been postulated that TLR10, which is encoded by a highly polymorphic gene, may be a potential asthma candidate gene (Lazarus *et al*., 2004). The most recently identified member of the TLR family is TLR11. In mice TLR11 responds to a surface-exposed factor on uropathogenic *E. coli* and protozoan profilin (Zhang *et al*., 2004; Lauw

et al., 2005); however, full length TLR11 expression in humans is prevented due to a stop codon mutation.

9.4 Airway epithelial cells, PRR polymorphisms and inflammatory lung disease

Bacteria or viruses responsible for acute airway infections such as pneumonia, rhinitis or exacerbations of chronic obstructive pulmonary disease (COPD) can trigger and activate many TLRs expressed by respiratory epithelial cells. Ideally a rapid and effective innate immune response is mounted, leading to recovery, elimination of the infective agent and resolution of any tissue damage. Impaired TLR function due to inherited genetic defects may lead to more severe disease and more devastating sequelae such as sepsis. The incidence of sepsis is predominantly associated with Gram-negative infection (Wenzel, 1992). Mutations in the TLR4 gene, e.g. (Asp299Gly), have been identified that are associated with a decreased airway response to inhaled LPS and an increased risk of Gram-negative infection and sepsis (Agnese *et al*., 2002; Child *et al*., 2003; Schwartz, 2002).

A number of TLR polymorphisms have been investigated with respect to inflammatory lung disease. For example, TLR2 Arg753Gln is associated with an increased TB risk (Ogus *et al*., 2004), and is also a potential risk factor for staphylococcal infection. Thus, it may have implications in cystic fibrosis (CF), as *Staphylococcus aureus* is commonly found in the CF lung (Lorenz *et al*., 2000). Other organisms commonly involved in the pathogenesis of pulmonary inflammation in CF are *P. aeruginosa* and *Haemophilus influenza*. TLR5 392STOP enhances susceptibility to *L. pneumophila* (Hawn *et al*., 2003) while TLR6 Ser249Pro has been linked with asthma (Tantisira *et al*., 2004). The role of TLR4 and LPS in asthma appears highly dependent on additional factors and conflicting reports exist regarding the effect of the TLR4 Arg299Gly polymorphism on the overall incidence of asthma (Raby *et al*., 2002; Werner *et al*., 2003; Yang *et al*., 2004). Similar controversy exists regarding the role of TLR4 in infective tuberculosis (TB) (Branger *et al*., 2004; Shim *et al*., 2003).

9.5 TLR expression in airway epithelial cells

As one of the first lines of defence in innate immunity in the lung, many transmembrane PRRs such as the TLRs are appropriately exposed on the mucosal surface of the airway and, in contrast to other epithelia such as the gut, can be readily activated by superficial exposure to microbial factors. Data from a variety of studies indicates that mRNA for all TLRs is expressed by airway epithelial cell lines (Becker *et al*., 2000; Greene *et al*., 2005; Muir *et al*., 2004). Studies using tracheal, bronchial and alveolar type II cell lines or primary respiratory tract cultures have also studied TLR distribution. It is now apparent that TLR subcellular expression differs between epithelial cells and immune cells. TLR2 protein is localized to the apical surface of these cells, whereas TLR4 and TLR5 have a more basolateral distribution (Adamo *et al*., 2004; Hertz *et al*., 2003; Muir *et al*., 2004). TLR4 appears to reside intracellularly in primary bronchial epithelial cells (Guillot *et al*., 2004b). TLRs 7, 8 and 9 reside in endosomes in macrophages and dendritic cells (Latz *et al*., 2004). Cell surface expression of TLR9 has been detected by fluorescence microscopy on a CF tracheal epithelial cell line and by flow cytometry on both immortalized and differentiated

primary airway epithelial cells (Greene *et al*., 2005; Platz *et al*., 2004). In vivo both TLR4 and TLR2 surface expression on alveolar type II cells has been reported (Armstrong *et al*., 2004; Droemann *et al*., 2003).

The predominant TLR expressed on the surface of bronchial and tracheal epithelial cells in vivo appears to be TLR2, with other TLRs (TLR3, TLR4, TLR5) residing mainly intracellularly or displaying only low-level surface expression. These TLRs can be mobilized to the cell surface following stimulation with microbial factors. For example TLR5 and TLR4 cell surface localization is promoted by flagellin and RSV infection, respectively (Adamo *et al*., 2004; Monick *et al*., 2003). TLR2, the *P. aeruginosa* pilus protein receptor asialo-GM1, caveolin-1, and the signalling molecules MyD88, IRAK-1, and TRAF6 exist in complexes within lipid rafts on the apical surface of airway epithelial cells after infection with *P. aeruginosa* (Soong *et al*., 2004). Similar multiprotein complexes involving TLR5 are evident in flagellin-treated airway epithelial cells (Adamo *et al*., 2004).

9.6 Intracellular signalling

Agonist-induced activation of TLRs leads to downstream signalling cascades resulting in the activation of pro-inflammatory gene transcription. Successful TLR-mediated alterations in gene expression are crucially dependent on the cytosolic TIR domain of TLRs (Akira and Hemmi, 2003; Yamamoto *et al*., 2004). The TIR domain serves as a scaffold for a series of protein–protein interactions specific to a number of signalling pathways, most notably those leading to the activation of NF-κB, mitogen-activated protein (MAP) kinases and PI3 kinase (Akira and Takeda, 2004).

An interesting feature of TLR signal transduction is that a conserved signalling pathway is activated by different TLRs (Bowie and O'Neill, 2000; O'Neill and Greene, 1998) mainly leading to transcription factor activation and changes in pro-inflammatory gene expression. Activation of NF-κB, AP1 and the MAP kinases JNK, p38 and ERK1/2 (Schroder *et al*., 2001) are other classical signals regulated by TLR signalling (Takeda and Akira, 2004). The pathway from TLRs leading to NF-κB involves recruitment of TIR domain-containing adaptor proteins which can associate with the cytosolic region of TLRs via TIR–TIR domain interactions. Activating functions have been assigned to four TIR adaptors: MyD88 (Medzhitov *et al*., 1998), Mal (Fitzgerald *et al*., 2001; Horng *et al*., 2001), TRIF (Oshiumi *et al*., 2003a; Yamamoto *et al*., 2002) and TRAM (Fitzgerald *et al*., 2003; Oshiumi *et al*., 2003b; Yamamoto *et al*., 2003b). A fifth TIR adaptor, SARM, acts as a negative regulator of TRIF-dependent TLR signalling (Carty *et al*., 2006).

Of all the TLRs, TLR4 is unique. In order to signal effectively it relies on two accessory proteins: MD-2, a soluble glycoprotein on the outer surface of the cell membrane (Nagai *et al*., 2002); and CD14, a glycophosphatidyl inositol-anchored receptor which binds to LPS–LPS-binding protein complexes (Chow *et al*., 1999). Intracellular signalling by all TLRs, with the exception of TLR3, involves MyD88. Once recruited, MyD88 interacts with IL-1 receptor-associated kinase-4 (IRAK-4) (Suzuki *et al*., 2002) and transduces the signal via IRAK-1, and tumour necrosis factor receptor-associated factor 6 (TRAF6) to transforming growth factor-β-activated kinase-1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2. Next, a larger complex is formed with Ubc13 and Uev1A, catalysing the synthesis of a polyubiquitin chain on TRAF6 (Deng *et al*., 2000) and triggering phosphorylation and activation of TAK1. This leads to activation of the IκB kinase (IKK) complex, consisting of IKKα, IKKβ and

NEMO/IKKγ (Wang *et al*., 2001), with subsequent phosphorylation, ubiquitination and proteosomal degradation of IκB, and concomitant nuclear translocation of NF-κB.

TLRs 2 and 4 utilize both MyD88 and Mal, and other TLRs (TLR3 and TLR4) can engage TRIF and TRAM under certain circumstances. NF-κB activation by MyD88 and Mal occurs as described, while engagement of TRIF and TRAM by TLR3 or TLR4 can also trigger a non-canonical signalling pathway, leading to expression of the type I interferons, involving the alternative IKKs, TANK-binding kinase 1 (TBK1) and IKKε/IKKi. These pathways culminate in activation of interferon regulatory factor (IRF) 3 and 7 (Figure 9.2) (Fitzgerald *et al*., 2003; Kawai *et al*., 2001; Sharma *et al*., 2003; Yamamoto *et al*., 2003a) transcription factors that regulate expression of IFN-β and IFN-α. In turn, expression of genes such as IP-10 and RANTES are increased via activation of STAT1. Although LPS does not induce expression of RANTES from BEAS-2B airway epithelial cells (Guillot *et al*., 2004b), TLR3

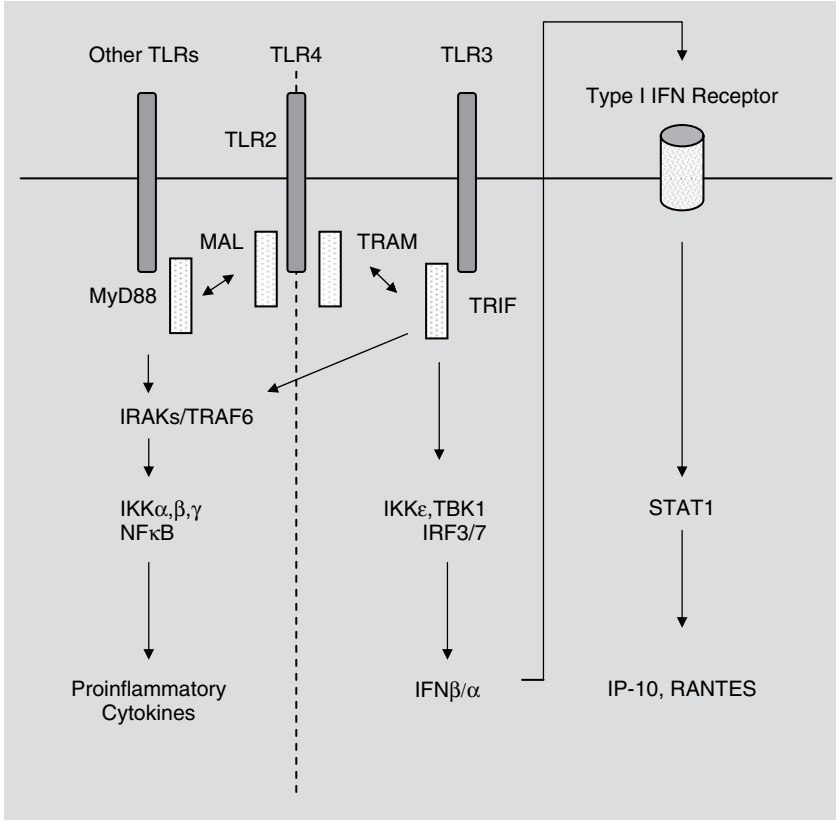


Figure 9.2 TLR signalling pathways. Triggering of TLRs promotes interaction between the TIR domains of TLRs and MyD88, Mal, Tram or TRIF as indicated. TLR2 and TLR4, or TLR3 activate the IKK complex via MyD88/Mal, or TRIF, respectively, leading to classical NF-κB activation. TLR3 and TLR4 also activate IKKε and TBK1 via TRIF (and TRAM for TLR4) leading to IRF3 and IRF7 activation and production of interferon-β and -α. These are secreted and bind to the type I interferon receptor triggering STAT1 activation and induction of interferon-inducible protein (IP-10) and RANTES

agonists have been shown to signal via TRIF to induce epithelial cell secretion of RANTES and IFN-β (Guillot *et al*., 2004a; Sha *et al*., 2004).

9.7 Functional responses to TLR activation in pulmonary epithelium

TLRs expressed by airway epithelial cells contribute to the pulmonary immune response by regulating the production and secretion of diffusible chemotatic molecules, antimicrobial peptides and cytokines, and by enhancing cell surface adhesion molecule expression (Adamo *et al*., 2004; Armstrong *et al*., 2004; Bachar *et al*., 2004; Becker *et al*., 2000; Gon *et al*., 2004; Greene *et al*., 2005; Guillot *et al*., 2004a, 2004b; Hertz *et al*., 2003; Homma *et al*., 2004; Jia *et al*., 2004; Monick *et al*., 2003; Muir *et al*., 2004; Platz *et al*., 2004; Sha *et al*., 2004; Soong *et al*., 2004; Wang *et al*., 2003).

As would be expected, a plethora of pro-inflammatory cytokines are regulated by TLR activation in airway epithelial cells. Tumour necrosis factor (TNF)-α and interleukin (IL)-6 can be induced by TLR2, TLR4 and TLR9 agonists to name but a few (Carroll *et al*., 2005; Greene *et al*., 2005; Homma *et al*., 2004; Monick *et al*., 2003). IL-8 is a potent neutrophil chemoattractant, and a widely studied reporter gene in TLR studies of airway epithelium. Many reports using immortalized and primary respiratory epithelial cells have demonstrated that an extensive repertoire of TLR agonists can regulate IL-8 gene transcription and protein production. The expression of other chemokines by airway epithelial cells is also regulated by TLR agonists that activate TLRs 2–5. MIP-3 expression is increased in response to zymosan, dsRNA, LPS, flagellin (Sha *et al*., 2004).

Human β-defensins (HBD) are antimicrobial peptides produced directly by epithelial cells. HBD2 expression can be induced in response to Gram-negative and Gram-positive bacteria or their components. TLR2 activation by bacterial lipoprotein enhances HBD2 expression in tracheobronchial epithelium (Hertz *et al*., 2003) while lipoteichoic acid and peptidoglycan are also known to induce TLR2-mediated increases in HBD2 expression in bronchial and alveolar airway epithelial cells (Homma *et al*., 2004; Wang *et al*., 2003). Agonists of TLR4 similarly regulate HBD2 expression in immortalized and primary airway epithelial cells (Jia *et al*., 2004).

An important event facilitating the transepithelial passage of leukocytes is enhanced integrin ligand expression. Surface expression of intercellular adhesion molecule 1 (ICAM-1) on airway epithelial is increased in response to triacylated lipopeptide, LPS and uCpG DNA (Greene *et al*., 2005). dsRNA and influenza virus A are also potent inducers of ICAM-1 in BEAS-2B epithelial cells (Guillot *et al*., 2004a).

9.8 Non-TLR transmembrane receptors

In addition to TLRs there are a number of other membrane-associated PRRs expressed by airway epithelial cells involved in pathogen recognition and the innate immune response in the lung. Lipoteichoic acid from *S. aureus* signals via platelet-activating factor receptor indirectly to the Epidermal Growth Factor receptor without the involvement of TLRs (Lemjabbar and Basbaum, 2002). Both *Streptococcus pneumoniae* and *H. influenzae* are also known to utilize the PAF receptor (Cundell *et al*., 1995; Swords *et al*., 2000). Another factor expressed

by *S. aureus*, the cell surface-anchored protein, protein A, can bind TNFRI and activate airway epithelial cells, while CD44 and CFTR have been implicated in airway epithelial responses to *Streptococcus pyogenes* and *P. aeruginosa*, respectively (Pier *et al*., 1996; Schrager *et al*., 1998). Complement receptors C3aR and C5aR are expressed on airway epithelium (Fregonese *et al*., 2005) and have important roles as anaphylatoxin receptors, most notably in asthma. Interestingly, complement itself has an immunological role in pneumonia. Type II alveolar epithelial cells can recognize *Klebsiella pneumoniae* via opsonic complement factor 3 (C3) fragments and the transmembrane receptor CD46 (de Astorza *et al*., 2004), without the involvement of CR1, CR2 or CR3. Thus non-classical transmembrane PRRs have an important role in microbial recognition by pulmonary epithelium. This phenomenon is not only limited to bacterial pathogens as the SARS coronavirus is known to bind angiotensin-converting enzyme 2 (ACE2) and utilize Type1a (AT1a) or Type 2 (AT2) angiotensin receptors expressed on the epithelial surface (Imai *et al*., 2005; Kuba *et al*., 2005).

9.9 Intracellular PRRs

The innate immune system comprises a dual system of surveillance encompassing extracellular sensing by transmembrane PRRs and intracellular sensing by cytosolic PRRs. The intracellular PRRs (icPRRs) are the cytosolic counterparts of TLRs and act as a second sentinel system that responds to invading pathogens. In the lung, organisms that can invade and replicate in airway epithelium include some streptococci (Cundell *et al*., 1995; Opitz *et al*., 2004), *L. pneumophila* (Cianciotto *et al*., 1995; Mody *et al*., 1993) and members of the *Chlamydia* genus (Jahn *et al*., 2000; Krull *et al*., 2005). Many viruses also have the capacity to replicate inside epithelial cells in the lung and these too can be sensed by a particular subset of icPRRs. Two families of icPRRs exist, the Nod-like receptors (NLRs) and the RNA RIG-like helicases (RLHs). These have roles in recognition of bacteria and viruses, respectively.

9.9.1 NLRs

The NLRs are modular proteins composed of a ligand recognition LRR carboxy terminal domain, a core Nod or NACHT region with ATPase activity, and an NH_2-terminal effector domain consisting of a baculoviral IAP repeat (Bir) or caspase-recruitment domain (CARD) (Figure 9.3). Other NLRs have an NH2-terminal pyrin domain. Over 23 members of the NLR family have been identified to date. Thus far, however, most is known about Nods 1 and 2, Naip and IPAF in airway epithelium.

Nod proteins sense the presence of intracellular muropeptides (Chamaillard *et al*., 2003; Girardin *et al*., 2003). Nod1 responds to muramyl tri- and tetra-peptide diaminopimelic acids; Nod2 senses muramyl dipeptide. Mutations in Nod1 are associated with increased susceptibility to asthma (Hysi *et al*., 2005), whilst Nod2 mutations are linked with granulomatous disease, in particular early-onset sarcoidosis (Kanazawa *et al*., 2005). Expression of both Nod1 and Nod2 has been detected in human lung epithelium (Opitz *et al*., 2004) with each implicated as having a role in pulmonary infection by *P. aeruginosa* and *Mycobacterium tuberculosis*, respectively (Ferwerda *et al*., 2005; Travassos *et al*., 2005).

Naip and IPAF sense intracellular bacterial flagellin independently of TLR5 (Franchi *et al*., 2006; Molofsky *et al*., 2006). Naip5 deficient mice have a higher than normal susceptibility

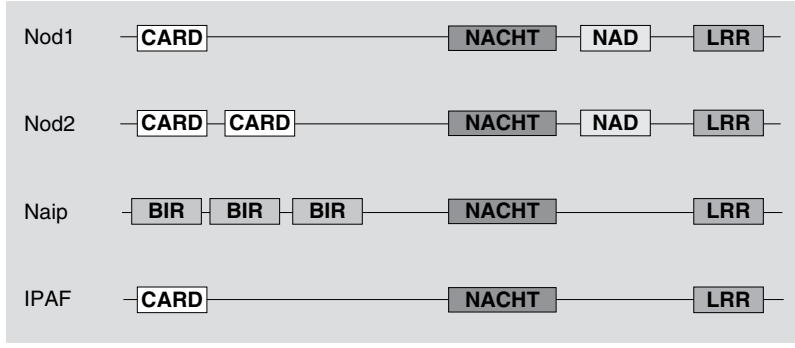


Figure 9.3 Structure of NLRs. NLRs consist of an N-terminal CARD or BIR domain, a central NACHT domain and a C-terminal LRR domain. The Nods are also characterized by a NAD region (NACHT-associated domain)

to pneumonia following *Legionella* infection. Together with IPAF, Naip can respond to adventitious motile pathogens. Sensing by NLRs can lead to activation of NF-κB via RIP2 and the caspase-1 inflammasome (reviewed in Fritz *et al*., 2006).

9.9.2 RLHs

Retinoic acid-inducible protein I (RIG-I) and melanoma differentiation-associated gene 5 (Mda-5) are member of the RNA helicase family that survey and respond to cytosolic viruses. RIG-I reportedly recognizes flavo-, paramyxo-, ortho- and rhabdoviruses, while Mda-5 has been implicated in the antiviral defence against picornaviruses. The process of viral replication can generate dsRNA. Until recently both helicases were believed to recognize dsRNA and, accordingly, behave as cytosolic homologues of TLR3. It is now suggested that RIG-I specifically recognizes viral genomic ssRNA bearing uncapped 5′-triphosphates, a motif absent in viruses known to be detected by Mda-5 (Hornung *et al*., 2006; Pichlmair *et al*., 2006).

The antiviral response induces type I interferon production and it is known that expression of dominant-negative RIG-I in lung epithelial cells can inhibit IFNβ gene expression (Matikainen *et al*., 2006). Furthermore RIG-I-deficient mice readily succumb to influenza virus infection highlighting the importance of this icPRR in immune surveillance in the lung (Kato *et al*., 2006).

9.10 Host-derived PRR agonists

It is not only microbial agonists that can activate PRRs but also a number of host-derived factors. Endogenous molecules including proteases (neutrophil elastase), heat shock proteins (Hsp60, Hsp70 Gp96), surfactant protein A, tissue matrix components (fibrinogen, fibronectin), hyaluronan oligosaccharides and antimicrobial molecules (defensins, ROS) can behave as TLR4 and TLR2 agonists (Biragyn *et al*., 2002; Ohashi *et al.*, 2000; Okamura *et al*., 2001; Taylor *et al*., 2004; Vabulas *et al*., 2002a, 2002b; Frantz *et al*., 2001; Guillot *et al*., 2002; Walsh *et al*., 2001). Stimulation of TLRs by these agents suggests that a mechanism

exists whereby TLRs can recognize molecular patterns of displaced factors or inflammatory mediators, become activated and enhance the immune response. Exactly how these endogenous agonists, or indeed the microbial agonists, interact with TLRs is unknown, but it has been postulated that accessory proteins may facilitate the process (Chaudhuri *et al*., 2005). As yet, little is known regarding activation of non-TLR and icPRRs by host-derived factors.

9.11 Conclusion

As a major portal of entry for microbes, the lung and its pulmonary epithelium represent a key component of the innate immune system. Airborne pathogens encounter a number of efficient defence mechanisms designed to neutralize potential damage, prevent colonization and safeguard against invasion by pathogens. The existence of both membrane-associated and intracellular PRRs equips the epithelium with a number of non-redundant mechanisms to control microbial infection and modulation of PRR function has obvious important implications for a variety of inflammatory lung diseases and the exacerbations thereof. Enhancing PRR responses using targeted approaches directed at TLR3 or RNA helicases, for example, could potentially accelerate anti-viral responses, while promoting uCpG/TLR9 signalling is known to enhance the Th1 response (Krug *et al*., 2001). On the other hand suppression of responses, by the use of neutralizing antibodies or inhibitors of intracellular signalling, may serve to reduce excessive inflammation in chronic inflammatory lung diseases. Thus, new therapeutics designed to selectively activate or inhibit PRR function specifically and reversibly represent powerful tools for the prevention and treatment of inflammatory diseases in the lung.

References

Adamo, R., Sokol, S., Soong, G., Gomez, M.I., and Prince, A. (2004) *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *Am J Respir Cell Mol Biol* **30**: 627–634.

Agnese, D.M., Calvano, J.E., Hahm, S.J., Coyle, S.M., Corbett, S.A., Calvano, S.E., and Lowry, S.F. (2002) Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis* **186**: 1522–1525.

Akira, S., and Hemmi, H. (2003) Recognition of pathogen-associated molecular patterns by TLR family. *Immunol Lett* **85**: 85–95.

Akira, S., and Takeda, K. (2004) Toll-like receptor signalling. *Nat Rev Immunol* **4**: 499–511.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**: 732–738.

Armstrong, L., Medford, A.R., Uppington, K.M., Robertson, J., Witherden, I.R., Tetley, T.D., and Millar, A.B. (2004) Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. *Am J Respir Cell Mol Biol* **31**: 241–245.

Bachar, O., Adner, M., Uddman, R., and Cardell, L.O. (2004) Toll-like receptor stimulation induces airway hyper-responsiveness to bradykinin, an effect mediated by JNK and NF-kappa B signaling pathways. *Eur J Immunol* **34**: 1196–1207.

Becker, M.N., Diamond, G., Verghese, M.W., and Randell, S.H. (2000) CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem* **275**: 29731–29736.

Bell, J.K., Mullen, G.E., Leifer, C.A., Mazzoni, A., Davies, D.R., and Segal, D.M. (2003) Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol* **24**: 528–533.

Biragyn, A., Ruffini, P.A., Leifer, C.A., Klyushnenkova, E., Shakhov, A., Chertov, O., Shirakawa, A.K., Farber, J.M., Segal, D.M., Oppenheim, J.J., and Kwak, L.W. (2002) Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **298**: 1025–1029.

Bowie, A., and O'Neill, L.A. (2000) The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J Leukoc Biol* **67**: 508–514.

Branger, J., Leemans, J.C., Florquin, S., Weijer, S., Speelman, P., and Van Der Poll, T. (2004) Toll-like receptor 4 plays a protective role in pulmonary tuberculosis in mice. *Int Immunol* **16**: 509–516.

Carroll, T.P., Greene, C.M., Taggart, C.C., Bowie, A.G., O'Neill, S.J., and McElvaney, N.G. (2005) Viral inhibition of IL-1- and neutrophil elastase-induced inflammatory responses in bronchial epithelial cells. *J Immunol* **175**: 7594–7601.

Carty, M., Goodbody, R., Schroder, M., Stack, J., Moynagh, P.N., and Bowie, A.G. (2006) The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat Immunol* **7**: 1074–1081.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M.A., Foster, S.J., Mak, T.W., Nunez, G., and Inohara, N. (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* **4**: 702–707.

Chaudhuri, N., Dower, S.K., Whyte, M.K., and Sabroe, I. (2005) Toll-like receptors and chronic lung disease. *Clin Sci (Lond)* **109**: 125–133.

Child, N.J., Yang, I.A., Pulletz, M.C., de Courcy-Golder, K., Andrews, A.L., Pappachan, V.J., and Holloway, J.W. (2003) Polymorphisms in Toll-like receptor 4 and the systemic inflammatory response syndrome. *Biochem Soc Trans* **31**: 652–653.

Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J., and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* **274**: 10689–10692.

Chu, H.W., Jeyaseelan, S., Rino, J.G., Voelker, D.R., Wexler, R.B., Campbell, K., Harbeck, R.J., and Martin, R.J. (2005) TLR2 signaling is critical for *Mycoplasma pneumoniae*-induced airway mucin expression. *J Immunol* **174**: 5713–5719.

Cianciotto, N.P., Stamos, J.K., and Kamp, D.W. (1995) Infectivity of *Legionella pneumophila* mip mutant for alveolar epithelial cells. *Curr Microbiol* **30**: 247–250.

Cundell, D.R., Gerard, N.P., Gerard, C., Idanpaan-Heikkila, I., and Tuomanen, E.I. (1995) *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* **377**: 435–438.

de Astorza, B., Cortes, G., Crespi, C., Saus, C., Rojo, J.M., and Alberti, S. (2004) C3 promotes clearance of *Klebsiella pneumoniae* by A549 epithelial cells. *Infect Immun* **72**: 1767–1774.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z.J. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**: 351–361.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**: 1529–1531.

Droemann, D., Goldmann, T., Branscheid, D., Clark, R., Dalhoff, K., Zabel, P., and Vollmer, E. (2003) Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. *Histochem Cell Biol* **119**: 103–108.

Ferwerda, G., Girardin, S.E., Kullberg, B.J., Le Bourhis, L., de Jong, D.J., Langenberg, D.M., van Crevel, R., Adema, G.J., Ottenhoff, T.H., Van der Meer, J.W., and Netea, M.G. (2005) NOD2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. *PLoS Pathog* **1**: 279–285.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jefferies, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T., McMurray, D., Smith, D.E., Sims, J.E., Bird, T.A., and O'Neill, L.A. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**: 78–83.

Fitzgerald, K.A., Rowe, D.C., Barnes, B.J., Caffrey, D.R., Visintin, A., Latz, E., Monks, B., Pitha, P.M., and Golenbock, D.T. (2003) LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* **198**: 1043–1055.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.D., Ozoren, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E.P., and Nunez, G. (2006) Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol* **7**: 576–582.

Frantz, S., Kelly, R.A., and Bourcier, T. (2001) Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *J Biol Chem* **276**: 5197–5203.

Fregonese, L., Swan, F.J., van Schadewijk, A., Dolhnikoff, M., Santos, M.A., Daha, M.R., Stolk, J., Tschernig, T., Sterk, P.J., Hiemstra, P.S., Rabe, K.F., and Mauad, T. (2005) Expression of the anaphylatoxin receptors C3aR and C5aR is increased in fatal asthma. *J Allergy Clin Immunol* **115**: 1148–1154.

Fritz, J.H., Ferrero, R.L., Philpott, D.J., and Girardin, S.E. (2006) Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* **7**: 1250–1257.

Gay, N.J., and Keith, F.J. (1991) Drosophila Toll and IL-1 receptor. *Nature* **351**: 355–356.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., Coyle, A.J., DiStefano, P.S., Bertin, J., Sansonetti, P.J., and Philpott, D.J. (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* **300**: 1584–1587.

Gomi, K., Kawasaki, K., Kawai, Y., Shiozaki, M., and Nishijima, M. (2002) Toll-like receptor 4-MD-2 complex mediates the signal transduction induced by flavolipin, an amino acid-containing lipid unique to *Flavobacterium meningosepticum. J Immunol* **168**: 2939–2943.

Gon, Y., Asai, Y., Hashimoto, S., Mizumura, K., Jibiki, I., Machino, T., Ra, C., and Horie, T. (2004) A20 inhibits toll-like receptor 2- and 4-mediated interleukin-8 synthesis in airway epithelial cells. *Am J Respir Cell Mol Biol* **31**: 330–336.

Greene, C.M., Carroll, T.P., Smith, S.G., Taggart, C.C., Devaney, J., Griffin, S., O'Neill S, J., and McElvaney, N.G. (2005) TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol* **174**: 1638–1646.

Guillot, L., Balloy, V., McCormack, F.X., Golenbock, D.T., Chignard, M., and Si-Tahar, M. (2002) Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J Immunol* **168**: 5989–5992.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M., and Si-Tahar, M. (2004a) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem.*

Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M., and Si-Tahar, M. (2004b) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem* **279**: 2712–2718.

Hasan, U., Chaffois, C., Gaillard, C., Saulnier, V., Merck, E., Tancredi, S., Guiet, C., Briere, F., Vlach, J., Lebecque, S., Trinchieri, G., and Bates, E.E. (2005) Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* **174**: 2942–2950.

Hawn, T.R., Verbon, A., Lettinga, K.D., Zhao, L.P., Li, S.S., Laws, R.J., Skerrett, S.J., Beutler, B., Schroeder, L., Nachman, A., Ozinsky, A., Smith, K.D., and Aderem, A. (2003) A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J Exp Med* **198**: 1563–1572.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**: 1099–1103.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**: 1526–1529.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**: 740–745.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* **3**: 196–200.

Hertz, C.J., Wu, Q., Porter, E.M., Zhang, Y.J., Weismuller, K.H., Godowski, P.J., Ganz, T., Randell, S.H., and Modlin, R.L. (2003) Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol* **171**: 6820–6826.

Hewson, C.A., Jardine, A., Edwards, M.R., Laza-Stanca, V., and Johnston, S.L. (2005) Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. *J Virol* **79**: 12273–12279.

Homma, T., Kato, A., Hashimoto, N., Batchelor, J., Yoshikawa, M., Imai, S., Wakiguchi, H., Saito, H., and Matsumoto, K. (2004) Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. *Am J Respir Cell Mol Biol* **31**: 463–469.

Honko, A.N., and Mizel, S.B. (2005) Effects of flagellin on innate and adaptive immunity. *Immunol Res* **33**: 83–101.

Horng, T., Barton, G.M., and Medzhitov, R. (2001) TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol* **2**: 835–841.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., Endres, S., and Hartmann, G. (2006) 5′-Triphosphate RNA is the ligand for RIG-I. *Science* **314**: 994–997.

Hysi, P., Kabesch, M., Moffatt, M.F., Schedel, M., Carr, D., Zhang, Y., Boardman, B., von Mutius, E., Weiland, S.K., Leupold, W., Fritzsch, C., Klopp, N., Musk, A.W., James, A., Nunez, G., Inohara, N., and Cookson, W.O. (2005) NOD1 variation, immunoglobulin E and asthma. *Hum Mol Genet* **14**: 935–941.

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., Yang, P., Sarao, R., Wada, T., Leong-Poi, H., Crackower, M.A., Fukamizu, A., Hui, C.C., Hein, L., Uhlig, S., Slutsky, A.S., Jiang, C., and Penninger, J.M. (2005) Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* **436**: 112–116.

Jahn, H.U., Krull, M., Wuppermann, F.N., Klucken, A.C., Rosseau, S., Seybold, J., Hegemann, J.H., Jantos, C.A., and Suttorp, N. (2000) Infection and activation of airway epithelial cells by *Chlamydia pneumoniae*. *J Infect Dis* **182**: 1678–1687.

Jia, H.P., Kline, J.N., Penisten, A., Apicella, M.A., Gioannini, T.L., Weiss, J., and McCray, P.B., Jr. (2004) Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol* **287**: L428–437.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., and Bauer, S. (2002) Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol* **3**: 499.

Kanazawa, N., Okafuji, I., Kambe, N., Nishikomori, R., Nakata-Hizume, M., Nagai, S., Fuji, A., Yuasa, T., Manki, A., Sakurai, Y., Nakajima, M., Kobayashi, H., Fujiwara, I., Tsutsumi, H., Utani, A., Nishigori, C., Heike, T., Nakahata, T., and Miyachi, Y. (2005) Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome. *Blood* **105**: 1195–1197.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.S., Reis e Sousa, C., Matsuura, Y., Fujita, T., and Akira, S. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**: 101–105.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P.F., Sato, S., Hoshino, K., and Akira, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* **167**: 5887–5894.

Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A.M., and Hartmann, G. (2001) Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol* **31**: 3026–3037.

Krull, M., Maass, M., Suttorp, N., and Rupp, J. (2005) *Chlamydophila pneumoniae*. Mechanisms of target cell infection and activation. *Thromb Haemost* **94**: 319–326.

Kuba, K., Imai, Y., Rao, S., Gao, H., Guo, F., Guan, B., Huan, Y., Yang, P., Zhang, Y., Deng, W., Bao, L., Zhang, B., Liu, G., Wang, Z., Chappell, M., Liu, Y., Zheng, D., Leibbrandt, A., Wada, T., Slutsky, A.S., Liu, D., Qin, C., Jiang, C., and Penninger, J.M. (2005) A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* **11**: 875–879.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J., and Finberg, R.W. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* **1**: 398–401.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K.A., Monks, B.G., Knetter, C.F., Lien, E., Nilsen, N.J., Espevik, T., and Golenbock, D.T. (2004) TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* **5**: 190–198.

Lauw, F.N., Caffrey, D.R., and Golenbock, D.T. (2005) Of mice and man: TLR11 (finally) finds profilin. *Trends Immunol* **26**: 509–511.

Lazarus, R., Raby, B.A., Lange, C., Silverman, E.K., Kwiatkowski, D.J., Vercelli, D., Klimecki, W.J., Martinez, F.D., and Weiss, S.T. (2004) TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am J Respir Crit Care Med* **170**: 594–600.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**: 973–983.

Lemjabbar, H., and Basbaum, C. (2002) Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* in epithelial cells. *Nat Med* **8**: 41–46.

Lopez-Boado, Y.S., Cobb, L.M., and Deora, R. (2005) *Bordetella bronchiseptica* flagellin is a proinflammatory determinant for airway epithelial cells. *Infect Immun* **73**: 7525–7534.

Lorenz, E., Mira, J.P., Cornish, K.L., Arbour, N.C., and Schwartz, D.A. (2000) A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect Immun* **68**: 6398–6401.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R., and Golenbock, D.T. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* **100**: 1966–1971.

Matikainen, S., Siren, J., Tissari, J., Veckman, V., Pirhonen, J., Severa, M., Sun, Q., Lin, R., Meri, S., Uze, G., Hiscott, J., and Julkunen, I. (2006) Tumor necrosis factor alpha enhances influenza A virus-induced expression of antiviral cytokines by activating RIG-I gene expression. *J Virol* **80**: 3515–3522.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C.A., Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* **2**: 253–258.

Mody, C.H., Paine, R., 3rd, Shahrabadi, M.S., Simon, R.H., Pearlman, E., Eisenstein, B.I., and Toews, G.B. (1993) *Legionella pneumophila* replicates within rat alveolar epithelial cells. *J Infect Dis* **167**: 1138–1145.

Molofsky, A.B., Byrne, B.G., Whitfield, N.N., Madigan, C.A., Fuse, E.T., Tateda, K., and Swanson, M.S. (2006) Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J Exp Med* **203**: 1093–1104.

Monick, M.M., Yarovinsky, T.O., Powers, L.S., Butler, N.S., Carter, A.B., Gudmundsson, G., and Hunninghake, G.W. (2003) Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* **278**: 53035–53044.

Muir, A., Soong, G., Sokol, S., Reddy, B., Gomez, M.I., Van Heeckeren, A., and Prince, A. (2004) Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol* **30**: 777–783.

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* **3**: 667–672.

Ogus, A.C., Yoldas, B., Ozdemir, T., Uguz, A., Olcen, S., Keser, I., Coskun, M., Cilli, A., and Yegin, O. (2004) The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J* **23**: 219–223.

Ohashi, K., Burkart, V., Flohe, S., and Kolb, H. (2000) Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* **164**: 558–561.

Okamura, Y., Watari, M., Jerud, E.S., Young, D.W., Ishizaka, S.T., Rose, J., Chow, J.C., and Strauss, J.F., 3rd (2001) The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem* **276**: 10229–10233.

O'Neill, L.A. (2002) Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. *Curr Top Microbiol Immunol* **270**: 47–61.

O'Neill, L.A., and Greene, C. (1998) Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J Leukoc Biol* **63**: 650–657.

Opitz, B., Puschel, A., Schmeck, B., Hocke, A.C., Rosseau, S., Hammerschmidt, S., Schumann, R.R., Suttorp, N., and Hippenstiel, S. (2004) Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae. J Biol Chem* **279**: 36426–36432.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003a) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* **4**: 161–167.

Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M., and Seya, T. (2003b) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J Biol Chem* **278**: 49751–49762.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* **314**: 997–1001.

Pier, G.B., Grout, M., Zaidi, T.S., Olsen, J.C., Johnson, L.G., Yankaskas, J.R., and Goldberg, J.B. (1996) Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science* **271**: 64–67.

Platz, J., Beisswenger, C., Dalpke, A., Koczulla, R., Pinkenburg, O., Vogelmeier, C., and Bals, R. (2004) Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *J Immunol* **173**: 1219–1223.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**: 2085–2088.

Raby, B.A., Klimecki, W.T., Laprise, C., Renaud, Y., Faith, J., Lemire, M., Greenwood, C., Weiland, K.M., Lange, C., Palmer, L.J., Lazarus, R., Vercelli, D., Kwiatkowski, D.J., Silverman, E.K., Martinez, F.D., Hudson, T.J., and Weiss, S.T. (2002) Polymorphisms in toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. *Am J Respir Crit Care Med* **166**: 1449–1456.

Rassa, J.C., Meyers, J.L., Zhang, Y., Kudaravalli, R., and Ross, S.R. (2002) Murine retroviruses activate B cells via interaction with toll-like receptor 4. *Proc Natl Acad Sci U S A* **99**: 2281–2286.

Ritter, M., Mennerich, D., Weith, A., and Seither, P. (2005) Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. *J Inflamm (Lond)* **2**: 16.

Rudd, B.D., Smit, J.J., Flavell, R.A., Alexopoulou, L., Schaller, M.A., Gruber, A., Berlin, A.A., and Lukacs, N.W. (2006) Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. *J Immunol* **176**: 1937–1942.

Sadikot, R.T., Blackwell, T.S., Christman, J.W., and Prince, A.S. (2005) Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* **171**: 1209–1223.

Sasu, S., LaVerda, D., Qureshi, N., Golenbock, D.T., and Beasley, D. (2001) *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res* **89**: 244–250.

Schmeck, B., Huber, S., Moog, K., Zahlten, J., Hocke, A.C., Opitz, B., Hammerschmidt, S., Mitchell, T.J., Kracht, M., Rosseau, S., Suttorp, N., and Hippenstiel, S. (2006) Pneumococci induced TLR- and Rac1-dependent NF-kappaB-recruitment to the IL-8 promoter in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **290**: L730–L737.

Schrager, H.M., Alberti, S., Cywes, C., Dougherty, G.J., and Wessels, M.R. (1998) Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A Streptococcus to CD44 on human keratinocytes. *J Clin Invest* **101**: 1708–1716.

Schroder, N.W., Pfeil, D., Opitz, B., Michelsen, K.S., Amberger, J., Zahringer, U., Gobel, U.B., and Schumann, R.R. (2001) Activation of mitogen-activated protein kinases p42/44, p38, and stress-activated protein kinases in myelo-monocytic cells by Treponema lipoteichoic acid. *J Biol Chem* **276**: 9713–9719.

Schwartz, D.A. (2002) TLR4 and LPS hyporesponsiveness in humans. *Int J Hyg Environ Health* **205**: 221–227.

Sha, Q., Truong-Tran, A.Q., Plitt, J.R., Beck, L.A., and Schleimer, R.P. (2004) Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* **31**: 358–364.

Sharma, S., tenOever, B.R., Grandvaux, N., Zhou, G.P., Lin, R., and Hiscott, J. (2003) Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**: 1148–1151.

Shim, T.S., Turner, O.C., and Orme, I.M. (2003) Toll-like receptor 4 plays no role in susceptibility of mice to *Mycobacterium tuberculosis* infection. *Tuberculosis (Edinb)* **83**: 367–371.

Soong, G., Reddy, B., Sokol, S., Adamo, R., and Prince, A. (2004) TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J Clin Invest* **113**: 1482–1489.

Suzuki, N., Suzuki, S., Duncan, G.S., Millar, D.G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J.M., Wesche, H., Ohashi, P.S., Mak, T.W., and Yeh, W.C. (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* **416**: 750–756.

Swords, W.E., Buscher, B.A., Ver Steeg Ii, K., Preston, A., Nichols, W.A., Weiser, J.N., Gibson, B.W., and Apicella, M.A. (2000) Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol Microbiol* **37**: 13–27.

Takeda, K., and Akira, S. (2004) TLR signaling pathways. *Semin Immunol* **16**: 3–9.

Tantisira, K., Klimecki, W.T., Lazarus, R., Palmer, L.J., Raby, B.A., Kwiatkowski, D.J., Silverman, E., Vercelli, D., Martinez, F.D., and Weiss, S.T. (2004) Toll-like receptor 6 gene (TLR6): single-nucleotide polymorphism frequencies and preliminary association with the diagnosis of asthma. *Genes Immun* **5**: 343–346.

Taylor, K.R., Trowbridge, J.M., Rudisill, J.A., Termeer, C.C., Simon, J.C., and Gallo, R.L. (2004) Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* **279**: 17079–17084.

Travassos, L.H., Carneiro, L.A., Girardin, S.E., Boneca, I.G., Lemos, R., Bozza, M.T., Domingues, R.C., Coyle, A.J., Bertin, J., Philpott, D.J., and Plotkowski, M.C. (2005) Nod1 participates in the innate immune response to *Pseudomonas aeruginosa. J Biol Chem* **280**: 36714–36718.

Vabulas, R.M., Ahmad-Nejad, P., Ghose, S., Kirschning, C.J., Issels, R.D., and Wagner, H. (2002a) HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J Biol Chem* **277**: 15107–15112.

Vabulas, R.M., Braedel, S., Hilf, N., Singh-Jasuja, H., Herter, S., Ahmad-Nejad, P., Kirschning, C.J., Da Costa, C., Rammensee, H.G., Wagner, H., and Schild, H. (2002b) The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. *J Biol Chem* **277**: 20847–20853.

Walsh, D.E., Greene, C.M., Carroll, T.P., Taggart, C.C., Gallagher, P.M., O'Neill, S.J., and McElvaney, N.G. (2001) Interleukin-8 up-regulation by neutrophil elastase is mediated by MyD88/IRAK/TRAF-6 in human bronchial epithelium. *J Biol Chem* **276**: 35494–35499.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**: 346–351.

Wang, X., Moser, C., Louboutin, J.P., Lysenko, E.S., Weiner, D.J., Weiser, J.N., and Wilson, J.M. (2002) Toll-like receptor 4 mediates innate immune responses to *Haemophilus influenzae* infection in mouse lung. *J Immunol* **168**: 810–815.

Wang, X., Zhang, Z., Louboutin, J.P., Moser, C., Weiner, D.J., and Wilson, J.M. (2003) Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. *Faseb J* **17**: 1727–1729.

Wenzel, R.P. (1992) Anti-endotoxin monoclonal antibodies – a second look. *N Engl J Med* **326**: 1151–1153.

Werner, M., Topp, R., Wimmer, K., Richter, K., Bischof, W., Wjst, M., and Heinrich, J. (2003) TLR4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol* **112**: 323–330.

Wetzler, L.M. (2003) The role of Toll-like receptor 2 in microbial disease and immunity. *Vaccine* **21** (Suppl 2): S55–60.

Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* **169**: 6668–6672.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003a) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301**: 640–643.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003b) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* **4**: 1144–1150.

Yamamoto, M., Takeda, K., and Akira, S. (2004) TIR domain-containing adaptors define the specificity of TLR signaling. *Mol Immunol* **40**: 861–868.

Yang, I.A., Barton, S.J., Rorke, S., Cakebread, J.A., Keith, T.P., Clough, J.B., Holgate, S.T., and Holloway, J.W. (2004) Toll-like receptor 4 polymorphism and severity of atopy in asthmatics. *Genes Immun* **5**: 41–45.

Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A., and Ghosh, S. (2004) A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* **303**: 1522–1526.

Zhang, Z., Louboutin, J.P., Weiner, D.J., Goldberg, J.B., and Wilson, J.M. (2005) Human airway epithelial cells sense *Pseudomonas aeruginosa* infection via recognition of flagellin by Toll-like receptor 5. *Infect Immun* **73**: 7151–7160.

10

Epithelial Antimicrobial Molecules

Pieter S. Hiemstra
Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands

Epithelial cells of the lung are exposed daily to large amounts of inhaled gases and substances, including lung pathogens. Despite this high exposure to pathogens, severe lung infections are rare in otherwise healthy individuals. This apparent discrepancy is partly explained by the innate immune activity of epithelial cells, that enables these cells to maintain a sterile environment even without recruiting phagocytes. Although this recruitment of phagocytes is an important defence mechanism, most microbial challenges are dealt with in the absence of such recruitment. A variety of mechanisms is employed by epithelial cells in host defence against infection, including barrier formation, mucociliary clearance and secretion of antimicrobial molecules. These molecules are especially important in the clearance of those micro-organisms that have penetrated the mucus layer and that have not been removed by mucociliary clearance (mucus and mucociliary clearance are discussed in Chapters 6 and 7). The nature of epithelial antimicrobial molecules is diverse, ranging from large proteins, such as lactoferrin, to small ones such as nitric oxide. In this chapter, an overview is provided of the various classes of antimicrobial molecules that are produced by the lung epithelium, including antimicrobial peptides and proteins, and reactive oxygen and nitrogen intermediates.

10.1 Antimicrobial peptides and proteins

Most organisms produce gene-encoded antimicrobial peptides and proteins (AMPs) as part of their innate defence against colonization and infection by microbial pathogens (Zasloff, 2002). AMPs are expressed in bacteria, plants and animals, indicating that this defence mechanism is evolutionary conserved. Several databases provide information about the hundreds of AMPs that have been characterized: AMSDb (http://www.bbcm.units.it/~tossi/pag1.htm), ANTIMIC (http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC) and APD (http://aps.unmc.edu/AP/main.html).

AMPs display marked broad-spectrum antimicrobial activity against a range of bacterial, fungal and (enveloped) viral pathogens, and neutralize microbial toxins such as

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

lipopolysaccharide (LPS) that is produced by Gram-negative bacteria (Bals and Hiemstra, 2004; Ganz, 2003; Zasloff, 2002). Most AMPs were isolated using a biochemical approach based on screening fractions for antimicrobial activity, but more recently genome-wide searches for molecular motifs that are characteristic for AMPs also have led to the discovery of novel AMPs. Despite their name, antimicrobial activity is certainly not their only activity. Recent studies have shown that AMPs also contribute to host defence through their effects on inflammation, immunity and wound repair.

Although the structure and mode of action of these molecules is diverse, many AMPs share cationic and amphipathic features that allow them to interact with the microbial membrane. The initial interaction between the positively charged AMPs and the negatively charged surface molecules on the target organism is thought to be dependent on electrostatic interactions. After this initial interaction with the microbial membrane, subsequent events lead to permeation of the membrane of the target organism, resulting in death. Several mechanisms have been described to mediate this membrane permeation, including formation of pores or channels in the target membrane (barrel-stave and aggregate channel models), and lysis through a carpet-like mechanism, where the microbial membrane is covered by a carpet of AMPs that result in formation of holes.

10.1.1 Families of AMPs

Based on their molecular mass, AMPs can be divided in antimicrobial peptides and proteins. In Table 10.1, an overview is provided of AMPs that are produced by the respiratory epithelium. The main families of antimicrobial peptides expressed in the lung are the defensins and the cathelicidins.

Table 10.1 Antimicrobial molecules secreted and/or generated by human respiratory epithelial cells

Antimicrobial peptides
α-defensins (HD-5)
β-defensins (hBD-1-4)
cathelicidin (hCAP-18/LL-37)
Antimicrobial proteins
lactoferrin (and derived peptides?)
lysozyme
cationic serine proteinase inhibitors (SLPI, elafin)
surfactant proteins
antimicrobial chemokines
C3a and C3a-desArg (proteolytic products of complement C3)
PLUNC proteins (?)
Small molecules
hypothiocyanite ($OSCN^-$) (from H_2O_2)
NO
peroxynitrite ($ONOO^-$) (from NO)

Defensins

Human defensins (HD) are small (3–5 kDa) cationic peptides that are non-glycosylated, and that are produced as preproproteins (Ganz, 2003; Schutte and McCray, 2002; Selsted

and Ouellette, 2005). The mature peptides contain a β-sheet structure and six cysteine residues that form three intramolecular disulfide bridges. Defensins are divided into three families based on their overall molecular structure and the spacing and connectivity of the disulfide bridges. In humans, members of two defensin families are expressed: α-defensins and β-defensins. The third family of circular minidefensins, the θ-defensins, are expressed in rhesus monkeys. Due to the presence of a stopcodon in the human gene, these defensins are not expressed in humans. Human neutrophil defensins (human neutrophil peptides [HNP] 1–4) are α-defensins expressed in neutrophils. The only α-defensin that appears to be expressed in airway epithelium is HD-5 (Frye *et al*., 2000). In contrast, airway epithelial cells express several β-defensins, including human β-defensin-1, -2, -3 and -4 (hBD-1, -2, -3 and -4). In addition, a splice variant of the human epididymis secretory protein (HE2) gene, HE2β1, encodes a cationic peptide that structurally resembles β-defensins, and that is expressed in bronchial epithelial cells (Jia *et al*., 2001).

Cathelicidins

Cathelicidins constitute a family of peptide antibiotics that contain a highly conserved signal sequence and pro-region, and marked heterogeneity in the C-terminal domain that encodes the mature cathelicidin peptide (Bals and Wilson, 2003; Tjabringa *et al*., 2005; Zanetti, 2004). Human (h)CAP-18 is the precursor protein of LL-37, which is the only cathelicidin peptide expressed in humans. hCAP-18/LL-37 is a cationic α-helical peptide that is mainly expressed in neutrophils, but also in epithelial cells and other cell types. Proteolytic processing of hCAP-18 results in the release of the active peptide. In neutrophils, this cleavage is mediated by proteinase 3, whereas prostate-derived gastricsin generates the related ALL-38 peptide from hCAP-18 that is produced in the epididymis. The proteolytic machinery that processes hCAP-18 in airway epithelial cells is unknown. Studies in skin have shown that kallikrein is involved in the process of generating small antimicrobial peptides derived from hCAP-18 that are present in skin and sweat (Yamasaki *et al*., 2006).

Lactoferrin

Lactoferrin is an approximately 80-kDa iron-binding glycoprotein that kills micro-organisms by sequestering iron that is required for microbial survival (Arnold *et al*., 1977). In addition, it also displays direct antimicrobial activity (Arnold *et al*., 1982) as demonstrated by small lactoferrin-derived peptides (lactoferricidin) that lack iron-binding properties. In addition to this antimicrobial activity against planktonic bacteria, lactoferrin inhibits formation of biofilms (Singh *et al*., 2002). Biofilms are structured communities of bacteria adherent to surfaces and encapsulated in a self-produced matrix, and bacteria in biofilms are largely resistant to antibiotic treatment and endogenous host defence mechanisms.

Lysozyme

Lysozyme was first discovered in nasal secretions based on its ‘bacteriolytic activity’ by Alexander Fleming (Fleming, 1922). It is a 14-kDa muramidase enzyme that degrades peptidoglycan leading to rapid cell death of Gram-positive bacteria. In addition to using enzymatic mechanisms, it also kills a range of bacteria via non-enzymatic mechanisms. A recent study in mice using muramidase-deficient recombinant lysozyme confirmed that the

enzymatic activity is not required for antimicrobial activity in vivo (Nash *et al*., 2006). In the lung, lysozyme is mainly produced by the serous cells in the submucosal glands and to a lesser extent by the surface epithelial cells (Dubin *et al*., 2004).

Cationic epithelial proteinase inhibitors – secretory leukocyte protease inhibitor (SLPI) and elafin

SLPI and elafin are cationic inhibitors of serine proteinases, such as neutrophil elastase that is released by neutrophils. This proteinase inhibitory activity led to their discovery, and was the main focus of research in the first years after their discovery. Later it was observed that these molecules also display antimicrobial activity, which is likely partly based on their cationic nature (Hiemstra *et al*., 1996; Simpson *et al*., 1999; Williams *et al*., 2006).

Surfactant proteins

Surfactant proteins (SP) A and SP-D are relatively abundant in respiratory secretions, and contribute to host defence by increasing uptake of micro-organisms by phagocytes, aggregation of micro-organisms and by direct antimicrobial activity (Wright, 2005). These surfactant proteins are produced by type II alveolar cells, but also by airway cells (Clara cells and cells of the submucosal glands). For a detailed discussion of surfactant proteins and their antimicrobial activity, see Chapter 8.

Antimicrobial chemokines

Several chemokines, including chemokines that are produced by airway epithelial cells, have been shown to display antimicrobial activity. These include members of the interferon-inducible ELR-CXC chemokines family (Cole *et al*., 2001), as well as CCL20 (Starner *et al*., 2003). Both their cationic nature and structural homology with β-defensins have been implicated in this antimicrobial activity.

Fragments from activated complement component C3

Complement activation is known to result directly in microbial killing through the formation of the membrane-attack complex, a pore-like structure formed in the membrane of the target cell. Recently it also was shown that the biologically active fragments of C3, C3a and its degradation product C3a-desArg, which are formed during complement activation, are directly antimicrobial to a range of bacteria (Nordahl *et al*., 2004).

PLUNC proteins

Palate lung nasal clone (PLUNC) was first discovered in nasal epithelium of the mouse embryo and in tracheal and bronchial epithelium of the adult mouse lung. Subsequent studies demonstrated the presence of a family of PLUNC proteins that is also expressed in humans, and that can be divided into short (SPLUNC) and long (LPLUNC) PLUNC. SPLUNC1 is a major secretory product of airway epithelial cell cultures, and is mainly expressed in

mucous cells and ducts of the submucosal glands (Bingle *et al*., 2005). Although no direct antimicrobial activity has yet been described, their pattern of expression, their homology with LPS-binding proteins, such as bactericidal/permeability increasing protein (BPI), and their intracellular colocalization with bacteria (Zhou *et al*., 2006) is highly suggestive of a role in host defence against infection.

Peptidoglycan recognition proteins (PGLYRPs)

Peptidoglycan recognition proteins (PGLYRPs) form a class of proteins that bind peptidoglycan and that have recently been recognized as antimicrobial proteins (Dziarski and Gupta, 2006). Although the expression of one of these (PGLYRP-2) is increased by bacterial challenge of keratinocytes, their expression in respiratory epithelial cells has not yet been reported.

10.1.2 Regulation of epithelial expression of AMPs

Epithelial cells constitutively produce a range of AMPs as a baseline defence mechanism, although for some AMPs this baseline expression is very low. However, the expression of various AMPs is inducible, and a variety of stimuli, including pro-inflammatory cytokines, growth factors and a large range of microbial products, have been shown to increase epithelial expression of antimicrobial molecules (Figure 10.1). Indeed, during inflammation and repair processes, expression of AMPs has been found to be markedly increased.

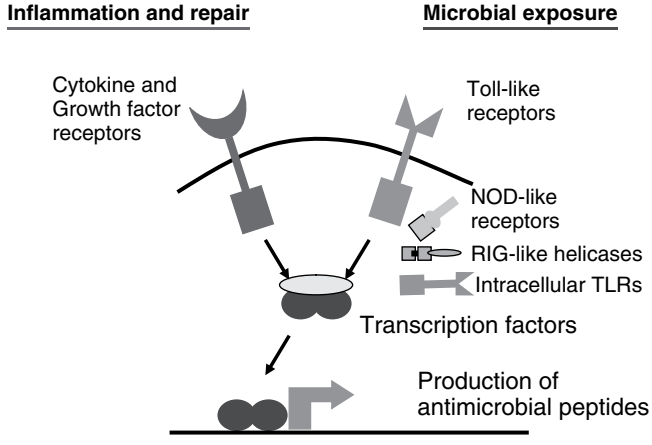


Figure 10.1 Regulation of the production of antimicrobial peptides and proteins during microbial exposure, inflammation and repair. A variety of membrane-bound and intracellular pattern-recognition receptors (involved in microbial recognition), cytokines and growth factors (involved in inflammation and repair) is involved in the regulation of production of antimicrobial peptides and proteins by epithelial cells. As discussed in the text, also other factors contribute to production of these epithelial antimicrobials

Epithelial cells employ various pattern-recognition receptors to sense microbial exposure, resulting in marked increases in the release of these AMPs (see Chapter 9 for a detailed review on microbial recognition by epithelial cells and the role of pattern-recognition receptors). Microbes may also indirectly increase expression of these effector molecules of innate immunity, by increasing the production of a range of pro-inflammatory cytokines from, for example, macrophages, that subsequently serve to increase the antimicrobial shield provided by AMPs. When the epithelial barrier is breached, a repair process follows that is associated with, and mediated by, local production of growth factors. These growth factors not only mediate wound repair, but also increase expression of AMPs and thus protect the underlying partly exposed tissue from infection.

Interestingly, a range of other stimuli also have been shown to mediate expression of AMPs. These include amino acids, butyrate and vitamin D. Expression of hCAP-18/LL-37 by phagocytes and epithelial cells has been shown to be regulated by vitamin D (Gombart *et al*., 2005; Wang *et al*., 2004). Microbial exposure may increase the local availability of active vitamin D3, as indicated by the recent observation that TLR2 ligands stimulate enzymatic conversion of inactive pro-vitamin D3 (25D3) to the active form of vitamin D3 (1,25 dihydroxyvitamin D_3; 1,25D3), which in turn results in increased hCAP-18/LL-37 expression in macrophages (Liu *et al*., 2006).

Conversely, other mediators decrease expression of AMPs. The Th2 cytokines IL-4 and IL-13 suppress expression of AMPs such as hBD-3 in cultured keratinocytes (Nomura *et al*., 2003). This suppression may at least partly explain the observation that expression of a range of AMPs in keratinocytes in patients with atopic dermatitis is decreased (De Jongh *et al*., 2005; Ong *et al*., 2002), which may underlie the increased susceptibility of patients with atopic dermatitis to skin infections. Such a mechanism may also be operative in the airway epithelium, as demonstrated by the ability of Th2 cytokines to suppress expression of hBD-2 in bronchial epithelial cells exposed to *Pseudomonas aeruginosa* and the deficiency of AMPs in mice with allergic airway inflammation (Beisswenger *et al*., 2006).

10.1.3 Activity of AMPs in airway secretions

Based on a quantitative analysis of AMPs present in airway secretions, it appears that lysozyme, lactoferrin and SLPI are by far the most abundant in these secretions and display synergistic antimicrobial activity (Travis *et al*., 1999). Nevertheless, the airway epithelium produces a range of other AMPs that are present at lower concentrations. These AMPs may act in concert (additive or synergistic) with lysozyme, lactoferrin, SLPI and other antimicrobial molecules that are distinct from AMPs. Furthermore, these AMPs may be active against a different range of micro-organisms, as well as display different activities in inflammation, immunity and wound repair, than do lysozyme, lactoferrin and SLPI.

AMPs that are produced by epithelial cells in the airway are released into the airway surface liquid (ASL). This ASL is composed of two layers: the periciliary layer around the cilia and microvilli, and the overlying mucus layer (Randell & Boucher, 2006). Using isolated components that are present in ASL of normal and inflamed lungs, it was shown that the activity of AMPs in ASL in vivo is likely tightly controlled. Several components restrict antimicrobial activity, including mucins (Felgentreff *et al*., 2006), bacterial polysaccharides released by lung pathogens (Herasimenka *et al*., 2005), and DNA and F-actin that are released from neutrophils and other inflammatory cells (Weiner *et al*., 2003). In addition, proteinases that are associated with airway inflammation inactivate selected AMPs: the

human β-defensins 2 and 3 (hBD-2 and -3) are inactivated by cysteine proteases (cathepsins B, L and S) that are present in increased concentrations in the lungs of patients with inflammatory lung diseases such as cystic fibrosis (Taggart *et al*., 2003).

In the past decade, many studies have focused on the possibility that AMPs may not act optimally in patients with cystic fibrosis (CF). These patients suffer from increased susceptibility to respiratory infections. It is the cycle of respiratory infections and inflammation that finally results in the respiratory failure that develops in end-stage disease. This increased susceptibility to infection appears to be related, in part, to a defective mucociliary clearance system due to dehydration of the mucosal surface of the lung. In addition, impairment of the activity of AMPs may restrict their activity in the CF lung. As discussed elsewhere in this section, a range of molecules that are present in increased concentrations in ASL in CF, including DNA from lysed inflammatory cells as well as cysteine proteinases, may impair the activity of AMPs. In addition, the ionic environment may limit this activity. Antimicrobial activity of most AMPs is markedly restricted at isotonic and hypertonic conditions, and optimal activity under laboratory conditions is observed at hypotonic conditions. It was originally shown that the ASL that is secreted by cultured airway epithelial cells from CF patients is deficient in antimicrobial activity as a result of increased salt concentrations (Smith *et al*., 1996). Subsequent studies showed that the activity of antimicrobial peptides such as β-defensins is salt-dependent and therefore may be reduced in ASL collected from CF airway epithelial cells (Goldman *et al*., 1997). Whether this is also a relevant mechanism in vivo remains to be determined, since the actual salt concentration of ASL in the lung is very difficult to determine because of the low volume of ASL in the lung, and the fragility of the mucosa during inflammation. Other studies revealed a salt-independent decrease in antimicrobial activity of ASL collected in a bronchial xenograft system in CF epithelial cells when compared to normal epithelial cells (Bals *et al*., 2001). Levels of various AMPs in the ASL of both study groups were comparable suggesting active suppression of antimicrobial activity in a salt-independent fashion. Therefore, various mechanisms may restrict the activity of AMPs in the lung in patients with CF, as well as other inflammatory and infectious lung disorders.

10.1.4 In vivo evidence for the role of AMPs in host defence in the lung

The importance of AMPs for host defence against infection has been demonstrated both in animal models and in human studies, showing that known clinical entities are associated with deficiencies (e.g. morbus Kostmann and Crohn's disease (Putsep *et al*., 2002; Wehkamp *et al*., 2005)) or polymorphisms (e.g. chronic obstructive pulmonary disease (COPD) (Matsushita *et al*., 2002)) in the genes encoding these peptides. Possibly, genetic variations contribute partly to defensin deficiency in selected individuals. Two studies have demonstrated an association between polymorphisms in the gene encoding human β-defensin-1 (*DEFB1*) and atopic asthma (Leung *et al*., 2006; Levy *et al*., 2005). Other possible genetic associations between lung disease and antimicrobial peptides include variable copy numbers in the β-defensin genes *DEFB4, DEFB103*, and *DEFB104* (Hollox *et al*., 2003). In cystic fibrosis, no association between *DEFB4* (encoding hBD-2) copy numbers and lung disease was reported. Whether lung disease is associated with aberrant copy numbers of these genes remains to be determined. In Japanese and Chinese populations, an association between two different hBD-1 (*DEFB1*) polymorphisms and COPD was observed (Hu *et al*., 2004; Matsushita *et al*., 2002). By contrast, no association between four different hBD-1

polymorphisms, or copy number variations in HNP-1/HNP-3, and lung function decline or infection was observed in smokers (Wallace *et al*., 2006). In addition to genetic variations in the genes encoding AMPs, genetic variation in other genes, such as pattern-recognition receptors, may affect AMP expression.

Not only human studies, but also various animal studies, have shown the role of AMPs in the protection against respiratory infections. Targeted deletion of the gene encoding mouse β-defensin-1 (mBD-1) resulted in delayed clearance of *Haemophilus influenzae* from the mouse lung (Moser *et al*., 2002). Conversely, overexpression of AMPs by gene transfer increases resistance to respiratory infections with, for example, *Pseudomonas aeruginosa*, as shown for hCAP-18/LL-37 and human elafin overexpression in mouse lung (Bals *et al*., 1999; Simpson *et al*., 2001), and for rat β-defensin-2 (rBD-2) in rat lung (Shu *et al*., 2006).

10.1.5 Non-antimicrobial functions of AMPs

Antimicrobial activity is not the only activity displayed by AMPs, although this activity was essential in the identification and characterization of most AMPs. Defensins and cathelicidins are AMPs that activate a range of host cells through the use of endogenous receptors (Oppenheim and Yang, 2005). The β-defensins hBD-1 and hBD-2 bind to the chemokine receptor CCR6, allowing them to attract immature dendritic cells and memory T-cells, and thereby bridge innate and adaptive immunity. LL-37 employs a range of cellular receptors for activation, including the formyl-peptide receptor-like 1 (FPRL1). This interaction is involved in the ability of LL-37 to attract neutrophils, eosinophils and monocytes, and to activate mast cells and to stimulate angiogenesis (Tjabringa *et al*., 2005, 2006). Furthermore, LL-37 may delay neutrophil apoptosis (Nagaoka *et al*., 2006) through tnteractions with FPRL1 and purinergic receptors, and modulates differentiation of dendritic cells via an unknown mechanism (Davidson *et al*., 2004). LL-37 is not only produced in limited amounts by epithelial cells, but also activates airway epithelial cells employing transactivation of the epidermal growth factor receptor resulting in increased production of, for example, IL-8 (Tjabringa *et al*., 2003). Neutrophil α-defensins also increase IL-8 production in lung epithelial cells, but this process appears to be dependent on activation of purinergic receptors (Khine *et al*., 2006).

AMPs have also been implicated in wound repair. Both neutrophil α-defensins and LL-37 mediate epithelial proliferation and wound repair processes, because they display growth factor activity (Aarbiou *et al*., 2004; Heilborn *et al*., 2003; Shaykhiev *et al*., 2005). The fact that many AMPs are present in high concentrations in wound fluids is explained by increased expression in injured epithelium, and the presence of inflammatory cells in wounds. These peptides may, thus, contribute to wound repair by their activity as growth factors, and by protecting the wound area from infection.

Other AMPs were discovered based on other activities, and only later found to display antimicrobial activity. SLPI and elafin are an example of this category of AMPs; they were discovered on the basis of their ability to inhibit serine proteinases, such as neutrophil elastase, and only years after their discovery were found to display antimicrobial activity. Fragments of complement factor C3 and surfactant proteins are other examples of this category.

These data show that AMPs not only act as endogenous antibiotics, but may also be regarded, for example, as chemoattractants, pro-inflammatory mediators and growth factors. The relative importance of these various activities remains to be determined, but the

observation that the local concentration of various AMPs in airway secretions is likely below or near their antimicrobial concentration indicates that these other activities may be important for our understanding of their role. In addition, these findings are highly relevant for the development of novel therapeutics based on administration of peptides derived from AMPs or enhancement of their production.

10.2 Reactive oxygen and nitrogen intermediates

The production of reactive oxygen intermediates and reactive nitrogen intermediates is considered as an effective mechanism employed by innate immune cells in the defence against microbial challenge. The production of these mediators by epithelial cells is discussed in more detail in Chapter 16. The overview provided in this chapter is focused on their antimicrobial function.

10.2.1 Reactive oxygen intermediates

Reactive oxygen intermediates (ROI) are an essential element of innate immunity and their formation has been well characterized in phagocytes. The phagocyte NADPH oxidase (*PhoX*), an enzymatic system to generate superoxide, is a central component of the antimicrobial function of neutrophils and macrophages. Hydrogen peroxide (H_2O_2) is produced from superoxide, and this H_2O_2 is converted to the antimicrobial hypochlorous acid (HOCl) by myeloperoxidase. Epithelial cells do not express myeloperoxidase, but the submucosal glands do secrete the homologue lactoperoxidase (LPO) that has recently been shown to contribute to formation of reactive oxygen intermediates in the airway surface liquid (ASL) (Conner *et al*., 2002) (Figure 10.2). LPO uses H_2O_2 to cause oxidation of thiocyanate

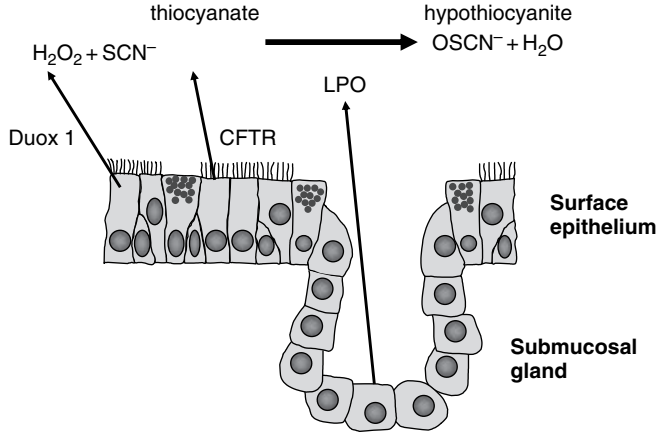


Figure 10.2 Role of reactive oxygen intermediates in the killing of micro-organisms by epithelial cells. The proposed role of Duox1 in generation of hydrogen peroxide (H_2O_2) and of the chloride channel CFTR (that is defective in cystic fibrosis) in transport of thiocyanate to the apical surface of surface epithelial cells is shown. Lactoperoxidase (LPO) derived from the submucosal gland cells mediates the formation of hypothiocyanite from these compounds

(SCN^-) that is present in ASL, resulting in the generation of the strongly antimicrobial hypothiocyanite ($OSCN^-$). Until recently, it was not clear whether epithelial cells produce sufficient H_2O_2 to contribute to this antimicrobial system in ASL, but studies now have demonstrated expression of homologues of gp91, the catalytic subunit of the phagocyte NADPH oxidase *PhoX*, in airway epithelium (Geiszt *et al*., 2003). These epithelial dual oxidases Duox1 and Duox2 were found to act as H_2O_2 sources, and, thus, contribute to LPO-mediated antimicrobial mechanisms in ASL (Geiszt *et al*., 2003; Moskwa *et al*., 2007). In addition to LPO and H_2O_2, the third component required for this LPO system is SCN^-, that is derived from the circulation. It was demonstrated that the chloride channel CFTR is involved in transport of SCN^- to the apical surface of epithelial cells, and that this mechanism is deficient in epithelial cells from patients with CF, due to CFTR mutations. As a consequence, this oxidative antimicrobial system appears inactive in CF, and this deficiency may contribute to increased sensitivity to infection in CF (Moskwa *et al*., 2007).

10.2.2 Reactive nitrogen intermediates

Nitric oxide (NO) is a molecule with a variety of actions in the lung, including antimicrobial activity (reviewed in Bogdan *et al*., 2000; Ricciardolo *et al*., 2004). NO is produced by various cell types in the lung, including epithelial cells, endothelial cells and inflammatory cells (reviewed by Ricciardolo *et al*., 2004). Epithelial cells from the respiratory tract express three different isoforms of the enzyme nitric oxide synthase (NOS): eNOS (NOS III), nNOS (NOS I) and iNOS (NOS II). The inducible iNOS has been studied in particular detail in the epithelium. NO has broad-spectrum antimicrobial activity against bacteria, fungi and viruses. The antiviral activity of NO is partly explained by its ability to cause S-nitrosylation of viral cysteine proteases that are essential for viral replication (Saura *et al*., 1999). NO also interacts with a range of molecular targets in the lung resulting in the formation of active substances. One of the most important is the interaction of NO with superoxide anion, resulting in the formation of the highly antimicrobial and cytotoxic peroxynitrite ($ONOO^-$) (Radi *et al*., 1991).

Whereas NO may contribute to the antimicrobial action of epithelial cells, it may also block some of its antimicrobial activity. Indoleamine 2,3-dioxygenase (IDO) causes tryptophan depletion which serves as an antimicrobial mechanism. It was demonstrated that exogenous NO blocks both IDO expression in epithelial cells, and the bacteriostatic action of interferon-γ stimulated epithelial cells against *Staphylococcus aureus* (Hucke *et al*., 2004).

10.3 Concluding remarks

The airway epithelium can be considered as a rich source of antimicrobial molecules. In view of the strategic position of the airway epithelium at the interface between the environment and the underlying tissue, this production of antimicrobial molecules is important to prevent respiratory infections. The wide variety of molecules present in airway secretions and their various spectra of activities and synergistic and additive interactions is important to meet a wide range of microbial challenges. These molecules partly contribute through their direct antimicrobial activity, but also by activating host cells directly and thereby affecting inflammation, immunity and wound repair. Both the production of antimicrobial molecules and their activity is subject to dynamic regulation, thus allowing the host to respond adequately to

microbial exposure. The increasing number of studies in specific patient groups, as demonstrated, for example, by studies in cystic fibrosis, have highlighted the role of antimicrobial molecules in host defence against infection, inflammation and immunity and in wound repair.

References

Aarbiou, J., Verhoosel, R. M., van Wetering, S., de Boer, W. I., van Krieken, J. H., Litvinov, S. V., Rabe, K. F., & Hiemstra, P. S. (2004) Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro, *Am J Respir Cell Mol Biol*, **30**, 193–201.

Arnold, R. R., Cole, M. F., & McGhee, J. R. (1977) A bactericidal effect for human lactoferrin, *Science*, **197**, 263–265.

Arnold, R. R., Russell, J. E., Champion, W. J., Brewer, M., & Gauthier, J. J. (1982) Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation, *Infect Immun*, **35**, 792–799.

Bals, R. & Hiemstra, P. S. (2004) Innate immunity in the lung: how epithelial cells fight against respiratory pathogens, *Eur Respira J*, **23**, 327–333.

Bals, R., Weiner, D. J., Moscioni, A. D., Meegalla, R. L., & Wilson, J. M. (1999) Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide, *Infect Immun*, **67**, 6084–6089.

Bals, R. & Wilson, J. M. (2003) Cathelicidins – a family of multifunctional antimicrobial peptides, *Cell Mol Life Sci*, **60**, 711–720.

Bals, R., Weiner, D. J., Meegalla, R. L., Accurso, F., & Wilson, J. M. (2001) Salt-independent abnormality of antimicrobial activity in cystic fibrosis airway surface fluid, *Am J Respir Cell Mol Biol*, **25**, 21–25.

Beisswenger, C., Kandler, K., Hess, C., Garn, H., Felgentreff, K., Wegmann, M., Renz, H., Vogelmeier, C., & Bals, R. (2006) Allergic airway inflammation inhibits pulmonary antibacterial host defense, *J Immunol*, **177**, 1833–1837.

Bingle, L., Cross, S. S., High, A. S., Wallace, W. A., Devine, D. A., Havard, S., Campos, M. A., & Bingle, C. D. (2005) SPLUNC1 (PLUNC) is expressed in glandular tissues of the respiratory tract and in lung tumours with a glandular phenotype, *J Pathol*, **205**, 491–497.

Bogdan, C., Rollinghoff, M., & Diefenbach, A. (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity, *Curr Opin Immun*, **12**, 64–76.

Cole, A. M., Ganz, T., Liese, A. M., Burdick, M. D., Liu, L., & Strieter, R. M. (2001) Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity, *J Immunol*, **167**, 623–627.

Conner, G. E., Salathe, M., & Forteza, R. (2002) Lactoperoxidase and hydrogen peroxide metabolism in the airway, *Am J Respir Crit Care Med*, **166**, S57–S61.

Davidson, D. J., Currie, A. J., Reid, G. S. D., Bowdish, D. M. E., MacDonald, K. L., Ma, R. C., Hancock, R. E. W., & Speert, D. P. (2004) The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization, *J Immunol*, **172**, 1146–1156.

De Jongh, G. J., Zeeuwen, P. L., Kucharekova, M., Pfundt, R., van der Valk, P. G., Blokx, W., Dogan, A., Hiemstra, P. S., van de Kerkhof, P. C., & Schalkwijk, J. (2005) High expression levels of keratinocyte antimicrobial proteins in psoriasis compared with atopic dermatitis, *J Invest Dermatol*, **125**, 1163–1173.

Dubin, R. F., Robinson, S. K., & Widdicombe, J. H. (2004) Secretion of lactoferrin and lysozyme by cultures of human airway epithelium, *Am J Physiol Lung Cell Mol Physiol*, **286**, L750–L755.

Dziarski, R. & Gupta, D. (2006) Mammalian PGRPs: novel antibacterial proteins, *Cell Microbiol*, **8**, 1059–1069.

Felgentreff, K., Beisswenger, C., Griese, M., Gulder, T., Bringmann, G., & Bals, R. (2006) The antimicrobial peptide cathelicidin interacts with airway mucus, *Peptides*, **27**, 3100–3106.

Fleming, A. (1922) On a remarkable bacteriolytic element found in tissues and secretions, *Proc Roy Soc London*, **93**, 306–310.

Frye, M., Bargon, J., Dauletbaev, N., Weber, A., Wagner, T. O., & Gropp, R. (2000) Expression of human alpha-defensin 5 (HD5) mRNA in nasal and bronchial epithelial cells, *J Clin Pathol*, **53**, 770–773.

Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity, *Nature Rev Immunol*, **3**, 710–720.

Geiszt, M., Witta, J., Baffi, J., Lekstrom, K., & Leto, T. L. (2003) Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense, *FASEB J*, **17**, 1502–1504.

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M., & Wilson, J. M. (1997) Human β-defensin-1 is a salt-sensitive antibiotic in the lung that is inactivated in cystic fibrosis, *Cell*, **88**, 553–560.

Gombart, A. F., Borregaard, N., & Koeffler, H. P. (2005) Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3, *FASEB J*, **19**, 1067–1077.

Heilborn, J. D., Nilsson, M. F., Kratz, G., Weber, G., Sorensen, O., Borregaard, N., & Stahle-Backdahl, M. (2003) The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium, *J Invest Dermatol*, **120**, 379–389.

Herasimenka, Y., Benincasa, M., Mattiuzzo, M., Cescutti, P., Gennaro, R., & Rizzo, R. (2005) Interaction of antimicrobial peptides with bacterial polysaccharides from lung pathogens, *Peptides*, **26**, 1127–1132.

Hiemstra, P. S., Maassen, R. J., Stolk, J., Heinzel-Wieland, R., Steffens, G. J., & Dijkman, J. H. (1996) Antibacterial activity of antileukoprotease, *Infect Immun*, **64**, 4520–4524.

Hollox, E. J., Armour, J. A., & Barber, J. C. (2003) Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster, *Am J Hum Genet*, **73**, 591–600.

Hu, R. C., Xu, Y. J., Zhang, Z. X., Ni, W., & Chen, S. X. (2004) Correlation of HDEFB1 polymorphism and susceptibility to chronic obstructive pulmonary disease in Chinese Han population, *Chin Med J (Engl)*, **117**, 1637–1641.

Hucke, C., MacKenzie, C. R., Adjogble, K. D. Z., Takikawa, O., & Daubener, W. (2004) Nitric oxide-mediated regulation of gamma interferon-induced bacteriostasis: inhibition and degradation of human indoleamine 2,3-dioxygenase, *Infect Immun*, **72**, 2723–2730.

Jia, H. P., Schutte, B. C., Schudy, A., Linzmeier, R., Guthmiller, J. M., Johnson, G. K., Tack, B. F., Mitros, J. P., Rosenthal, A., Ganz, T., & McCray, P. B., Jr. (2001) Discovery of new human beta-defensins using a genomics-based approach, *Gene*, **263**, 211–218.

Khine, A. A., Del Sorbo, L., Vaschetto, R., Voglis, S., Tullis, E., Slutsky, A. S., Downey, G. P., & Zhang, H. (2006) Human neutrophil peptides induce interleukin-8 production through the P2Y6 signaling pathway, *Blood*, **107**, 2936–2942.

Leung, T. F., Li, C. Y., Liu, E. K., Tang, N. L., Chan, I. H., Yung, E., Wong, G. W., & Lam, C. W. (2006 Asthma and atopy are associated with DEFB1 polymorphisms in Chinese children, *Genes Immun*, **7**, 59–64.

Levy, H., Raby, B. A., Lake, S., Tantisira, K. G., Kwiatkowski, D., Lazarus, R., Silverman, E. K., Richter, B., Klimecki, W. T., Vercelli, D., Martinez, F. D., & Weiss, S. T. (2005) Association of defensin beta-1 gene polymorphisms with asthma, *J Allergy Clin Immunol*, **115**, 252–258.

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., Ochoa, M. T., Schauber, J., Wu, K., Meinken, C., Kamen, D. L., Wagner, M., Bals, R., Steinmeyer, A., Zugel, U., Gallo, R. L., Eisenberg, D., Hewison, M., Hollis, B. W., Adams, J. S., Bloom, B. R., & Modlin, R. L. (2006) Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response, *Science*, **311**, 1770–1773.

Matsushita, I., Hasegawa, K., Nakata, K., Yasuda, K., Tokunaga, K., & Keicho, N. (2002) Genetic variants of human beta-defensin-1 and chronic obstructive pulmonary disease, *Biochem Biophysi Res Commun*, **291**, 17–22.

Moser, C., Weiner, D. J., Lysenko, E., Bals, R., Weiser, J. N., & Wilson, J. M. (2002) beta-Defensin 1 contributes to pulmonary innate immunity in mice, *Infect Immun*, **70**, 3068–3072.

Moskwa, P., Lorentzen, D., Excoffon, K. J. D. A., Zabner, J., McCray, P. B., Jr., Nauseef, W. M., Dupuy, C., & Banfi, B. (2007) A novel host defense system of airways is defective in cystic fibrosis, *Am J Respir Crit Care Med*, **175**, 174–183.

Nagaoka, I., Tamura, H., & Hirata, M. (2006) An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7, *J Immunol*, **176**, 3044–3052.

Nash, J. A., Ballard, T. N., Weaver, T. E., & Akinbi, H. T. (2006) The peptidoglycan-degrading property of lysozyme is not required for bactericidal activity in vivo, *J Immunol*, **177**, 519–526.

Nomura, I., Goleva, E., Howell, M. D., Hamid, Q. A., Ong, P. Y., Hall, C. F., Darst, M. A., Gao, B. F., Boguniewicz, M., Travers, J. B., & Leung, D. Y. M. (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes, *J Immunol*, **171**, 3262–3269.

Nordahl, E. A., Rydengard, V., Nyberg, P., Nitsche, D. P., Morgelin, M., Malmsten, M., Bjorck, L., & Schmidtchen, A. (2004) Activation of the complement system generates antibacterial peptides, *Proc Natl Acad Sci U S A*, **101**, 16879–16884.

Ong, P. Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R. L., & Leung, D. Y. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis, *N Engl J Med*, **347**, 1151–1160.

Oppenheim, J. J. & Yang, D. (2005) Alarmins: chemotactic activators of immune responses, *Curr Opin Immunol*, **17**, 359–365.

Putsep, K., Carlsson, G., Boman, H., & Andersson, M. (2002) Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study, *The Lancet*, **360**, 1144–1149.

Radi, R., Beckman, J. S., Bush, K. M., & Freeman, B. A. (1991) Peroxynitrite-induced membrane lipid-peroxidation – the cytotoxic potential of superoxide and nitric-oxide, *Arch Biochem Biophys*, **288**, 481–487.

Randell, S. H. & Boucher, R. C. (2006) Effective mucus clearance is essential for respiratory health, *American J Respir Cell Mol Biol*, **35**, 20–28.

Ricciardolo, F. L., Sterk, P. J., Gaston, B., & Folkerts, G. (2004) Nitric oxide in health and disease of the respiratory system, *Physiol Rev*, **84**, 731–765.

Saura, M., Zaragoza, C., McMillan, A., Quick, R. A., Hohenadl, C., Lowenstein, J. M., & Lowenstein, C. J. (1999) An antiviral mechanism of nitric oxide: inhibition of a viral protease, *Immunity*, **10**, 21–28.

Schutte, B. C. & McCray, P. B., Jr. (2002) beta-defensins in lung host defense, *Annu Rev Physiol*, **64**, 709–748.

Selsted, M. E. & Ouellette, A. J. (2005) Mammalian defensins in the antimicrobial immune response, *Nat Immunol*, **6**, 551–557.

Shaykhiev, R., Beisswenger, C., Kaendler, K., Senske, J., Puechner, A., Damm, T., Behr, J., & Bals, R. (2005) Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure, *Am J Physiol Lung Cell Mol Physiol*, **289**, L842–L848.

Shu, Q., Shi, Z., Zhao, Z. Y., Chen, Z., Yao, H. P., Chen, Q. X., Hoeft, A., Stuber, F., & Fang, X. M. (2006) Protection against *Pseudomonas aeruginosa* pneumonia and sepsis-induced lung injury by overexpression of beta-defensin-2 in rats, *Shock*, **26**, 365–371.

Simpson, A. J., Maxwell, A. I., Govan, J. R., Haslett, C., & Sallenave, J. M. (1999) Elafin (elastase-specific inhibitor) has anti-microbial activity against gram-positive and gram-negative respiratory pathogens, *FEBS Lett*, **452**, 309–313.

Simpson, A. J., Wallace, W. A., Marsden, M. E., Govan, J. R., Porteous, D. J., Haslett, C., & Sallenave, J. M. (2001) Adenoviral augmentation of elafin protects the lung against acute injury mediated by activated neutrophils and bacterial infection, *J Immunol*, **167**, 1778–1786.

Singh, P. K., Parsek, M. R., Greenberg, E. P., & Welsh, M. J. (2002) A component of innate immunity prevents bacterial biofilm development, *Nature*, **417**, 552–555.

Smith, J. J., Travis, S. M., Greenberg, E. P., & Welsh, M. J. (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid, *Cell*, **85**, 229–236.

Starner, T. D., Barker, C. K., Jia, H. P., Kang, Y., & McCray, P. B., Jr. (2003) CCL20 is an inducible product of human airway epithelia with innate immune properties, *Am J Respir Cell Mol Biol*, **29**, 627–633.

Taggart, C. C., Greene, C. M., Smith, S. G., Levine, R. L., McCray, P. B., Jr., O'Neill, S., & McElvaney, N. G. (2003) Inactivation of human beta-defensins 2 and 3 by elastolytic cathepsins, *J Immunol*, **171**, 931–937.

Tjabringa, G. S., Aarbiou, J., Ninaber, D. K., Drijfhout, J. W., Sorensen, O. E., Borregaard, N., Rabe, K. F., & Hiemstra, P. S. (2003) The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor, *J Immunol*, **171**, 6690–6696.

Tjabringa, G. S., Rabe, K. F., & Hiemstra, P. S. (2005) The human cathelicidin LL-37: a multifunctional peptide involved in infection and inflammation in the lung, *Pulm Pharmacol Ther*, **18**, 321–327.

Tjabringa, G. S., Ninaber, D. K., Drijfhout, J. W., Rabe, K. F., & Hiemstra, P. S. (2006) Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors, *Int Arch Allergy Immunol*, **140**, 103–112.

Travis, S. M., Conway, B. A., Zabner, J., Smith, J. J., Anderson, N. N., Singh, P. K., Greenberg, E. P., & Welsh, M. J. (1999) Activity of abundant antimicrobials of the human airway, *Am J Respir Cell Mol Biol*, **20**, 872–879.

Wallace, A. M., He, J. Q., Burkett, K. M., Ruan, J., Connett, J. E., Anthonisen, N. R., Pare, P. D., & Sandford, A. J. (2006) Contribution of alpha- and beta-defensins to lung function decline and infection in smokers: an association study, *Respir Res*, **7**, 76.

Wang, T. T., Nestel, F. P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., Tavera-Mendoza, L., Lin, R., Hanrahan, J. W., Mader, S., & White, J. H. (2004) Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression, *J Immunol*, **173**, 2909–2912.

Wehkamp, J., Salzman, N. H., Porter, E., Nuding, S., Weichenthal, M., Petras, R. E., Shen, B., Schaeffeler, E., Schwab, M., Linzmeier, R., Feathers, R. W., Chu, H., Lima, H., Jr., Fellermann, K., Ganz, T., Stange, E. F., & Bevins, C. L. (2005) Reduced Paneth cell alpha-defensins in ileal Crohn's disease, *Proc Natl Acad Sci U S A*, **102**, 18129–18134.

Weiner, D. J., Bucki, R., & Janmey, P. A. (2003) The antimicrobial activity of the cathelicidin LL37 is inhibited by F-actin bundles and restored by gelsolin, *Am J Respir Cell Mol Biol*, **28**, 738–745.

Williams, S. E., Brown, T. I., Roghanian, A., & Sallenave, J. M. (2006) SLPI and elafin: one glove, many fingers, *Clin Sci (Lond)*, **110**, 21–35.

Wright, J. R. (2005) Immunoregulatory functions of surfactant proteins, *Nat Rev Immunol*, **5**, 58–68.

Yamasaki, K., Schauber, J., Coda, A., Lin, H., Dorschner, R. A., Schechter, N. M., Bonnart, C., Descargues, P., Hovnanian, A., & Gallo, R. L. (2006) Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin, *FASEB J*, **20**, 2068–2080.

Zanetti, M. (2004) Cathelicidins, multifunctional peptides of the innate immunity, *J Leukoc Biol*, **75**, 39–48.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms, *Nature*, **415**, 389–395.

Zhou, H. D., Li, G. Y., Yang, Y. X., Li, X. L., Sheng, S. R., Zhang, W. L., & Zhao, J. (2006) Intracellular co-localization of SPLUNC1 protein with nanobacteria in nasopharyngeal carcinoma epithelia HNE1 cells depended on the bactericidal permeability increasing protein domain, *Mol Immunol*, **43**, 864–871.

11

The Epithelium and Immunoregulation

Angela Rate[1] and **John W. Upham**[2]
[1] *Telethon Institute for Child Health Research, Perth, Western Australia*
[2] *School of Medicine, University of Queensland, Princess Alexandra Hospital, Brisbane, Woolloongabba, Australia*

11.1 Introduction

The major function of the respiratory epithelium was once thought to be primarily that of a physical barrier. However, for a number of reasons, there can be little doubt regarding the importance of airway epithelial cells (AEC) in regulating many other processes including inflammatory responses seen in respiratory diseases. The airway epithelium is continuously exposed to a variety of inhaled and locally generated stimuli that are likely to have direct influences on the nature of the immune response to inhaled antigens and allergens and the development of inflammation of the airway and alveoli.

Although the epithelium has an important physical role in airway homeostasis, for example, by regulating the composition and nature of the airway surface liquid and through muco-ciliary clearance, the airway responses to injury highlight the complex nature of the interactions of AEC with a wide range of cells and processes. In recent years, the participation of AEC in immunoregulation has come under close scrutiny, and it is clear that cytokines and growth factors produced by AEC, as well as the array of molecules expressed on their surface, can contribute significantly to airway repair processes and to the regulation of a variety of immune responses (Holgate *et al*., 2000). Specifically, AEC interact with, and regulate, cells of the adaptive immune response (including T-cells, B-cells and dendritic cells) and, as will be discussed in this chapter, these interactions are integral in determining the immunological balance in the lung.

11.2 The pulmonary epithelium and dendritic cells

There is now clear evidence that local (airway) and systemic immune responses to inhaled antigen are coordinated such that dendritic cells (DC) in the airway epithelium can be

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

provoked to take up, process, and present antigens in situ. Alternatively, DC may differentiate and migrate to draining lymph nodes to present antigen to systemic T-cells (Hayday and Viney, 2000). The physical proximity of DC to AEC, and the range of proteins produced by AEC and DC with the potential for reciprocal regulatory effects, suggest a central role for interactions between AEC and DC in the airway responses to antigens.

11.2.1 Normal dendritic cell biology

Respiratory tract DC are recognized as having a vital role in the regulation of adaptive immune responses to inhaled foreign antigens. They are ubiquitous throughout the respiratory tract, forming a tight network of cells within the epithelium and submucosa of the conducting airway, the lung parenchyma and the nasal mucosa. DC are actively endocytic (Stumbles *et al*., 1998), making them ideally situated to sample environmental antigens and to function as 'sentinels of the immune system'.

Not only are DC major players in the initiation and amplification of immune responses, they also regulate the qualitative nature of these events, and exert a significant influence over Th1/Th2 polarization and the development of tolerance to inhaled innocuous antigens (Akbari *et al*., 2001; Banchereau and Steinman, 1998; Holt and Upham, 2004; Huh *et al*., 2003; Jahnsen *et al*., 2001; Lambrecht *et al*., 2000; McWilliam *et al*., 1996; van Rijt *et al*., 2005). Even under steady-state conditions, there is a continuous turnover of lung DC, with the recruitment of immature DC from circulating precursors, balanced by the exit of mature, antigen-loaded cells to regional lymph nodes. Such DC turnover increases dramatically in response to events occurring at the epithelial surface, especially exposure to inhaled irritants, particulate matter, microbial stimuli, and soluble antigens or allergen (Jahnsen *et al*., 2001; McWilliam *et al*., 1996; Upham *et al*., 2002).

The function of recently recruited DC is likely to be altered as a consequence of local tissue factors. Freshly isolated lung DC exhibit a poor capacity for antigen presentation and express only low levels of co-stimulatory molecules, but rapidly undergo spontaneous activation when cultured ex vivo, even in the absence of added stimuli. This suggests that the lung microenvironment provides inhibitory signals that counteract DC activation in vivo, and this influence may remain even after DC have left the epithelium and migrated to regional lymph nodes (Kalinski *et al*., 1998). Such a mechanism is likely to involve alveolar macrophages and AEC, and is thought to be important for maintaining immune homeostasis.

It would seem entirely appropriate for the influence of the lung milieu to become more 'permissive' toward DC activation in the face of certain inflammatory stimuli, once a threshold dose of stimulus has been applied. Whereas interstitial DC residing in non-lymphoid organs usually do not express DC activation markers or the nuclear transcription factor RelB (thought to play a key role in antigen presentation), activated DC in inflamed rheumatoid synovium express nuclear RelB (Pettit *et al*., 2000). Though it is likely that similar principles apply in the context of airway epithelial injury or inflammation, this has not been examined in lung disease.

11.2.2 Epithelial cell regulation of dendritic cells

Concomitant with antigen acquisition in the airway mucosa, DC may be particularly receptive to local signals derived from AEC, given the close proximity of these two cell types. The extent of cross-talk between epithelial cells (EC) and DC has been most closely examined in

the skin and gastro-intestinal tract. There is strong evidence from the dermatology literature that EC-derived factors modulate the differentiation and function of dermal DC and the specialized epidermal DC known as Langerhans cells (LC). Differentiation of skin LC appears to be dependent on transforming growth factor beta (TGFβ), as LC are absent from the epidermis in TGFβ knockout mice, and TGFβ appears to be necessary for the in vitro differentiation of monocytes and other DC precursors into LC (Borkowski *et al*., 1996; Geissmann *et al*., 1998). Moreover, blood-derived DC precursors give rise to typical epidermal LC when co-cultured with normal human keratinocytes (Regnier *et al*., 1998), and ligation of E-cadherin on the surface of DC actively suppresses their maturation (Riedl *et al*., 2000; Fujita *et al*., 2006).

In the gut, DC are able to open the tight junctions between EC, extending their processes between adjacent EC in order to sample bacteria from the gut lumen without compromising the integrity of the epithelial barrier (Rescigno *et al*., 2001). Formation of these trans-epithelial dendrites is dependent on the chemokine receptor CX3CR1 (fractalkine receptor) expressed on DC that interacts with its fractalkine ligand expressed by EC (Niess *et al*., 2005). EC regulate DC activation, though this varies depending on whether the epithelium is exposed to invasive or non-invasive bacteria.

There is less information available on the extent to which AEC regulate DC function. Exposure of lung epithelial cell lines to the protease allergen Der p 1 increases chemokine release and thereby facilitate DC chemotaxis (Pichavant *et al*., 2005). AEC can certainly express a variety of adhesion molecules and soluble mediators (see Table 11.1) through which they are likely to modulate DC function within the airway (Stick and Holt, 2003), though the relative importance of each of these mediators has not been examined in any detail.

Table 11.1 Potential mechanisms by which pulmonary epithelial cells might regulate DC function

Molecules expressed by lung epithelial cells	Effects on dendritic cells	Reference
TGFβ1	Selectively inhibits antigen presentation by DC, and DC/T-cell interactions	(Geissmann *et al*., 1999; Geissmann *et al*., 1998)
Prostaglandins	PGE_2 inhibits DC activation, favours Th2 responses PGD_2 alters DC migration and activation	(Gosset *et al*., 2003; Kalinski *et al*., 1998)
Nitric oxide	Inhibits maturation and antigen presentation	(Lane *et al*., 2004; Paolucci *et al*., 2000)
Thymic stromal lymphopoietin (TSLP)	Acts directly on DC to induce allergic inflammation by driving Th2 responses	(Soumelis *et al*., 2002; Ying *et al*., 2005)
IL-10	Inhibits antigen presentation, DC activation	(Moore *et al*., 2001)
Fractalkine	Formation of trans-epithelial dendrites	(Fujimoto *et al*., 2001; Niess *et al*., 2005)
Defensins	DC activation	(Biragyn *et al*., 2002)
Heat shock proteins	Inhibits DC differentiation from monocytes; induces immature DC maturation	(Kuppner *et al*., 2001)
Secretory leukocyte protease inhibitor (SLPI)	Inhibits NF-κB activation and impairs TLR2 and TLR4 responses in monocytic cells though specific effects on DC are not known	(Greene *et al*., 2004; Henriksen *et al*., 2004)

Table 11.1 (Continued)

Molecules expressed by lung epithelial cells	Effects on dendritic cells	Reference
Elafin	Inhibits NF-κB activation; increases the number and activation state of lung DC, leading to enhanced Th1 immune responses in an animal model	(Henriksen *et al*., 2004; Roghanian *et al*., 2006)
Vascular endothelial growth factor (VEGF)	Impairs DC maturation and antigen presentation to T-cells	(Laxmanan *et al*., 2005)
E-cadherin	Inhibits DC maturation and chemokine production; promotes binding of DC at sites of antigen deposition	(Fujita *et al*., 2006; Riedl *et al*., 2000) (Carayol *et al*., 2002)
Intercellular adhesion molecule 1 (ICAM-1)	Promotes DC activation upon binding to LFA-1	(Pichavant *et al*., 2006)
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Promotes DC differentiation from precursors and antigen presentation to T-cells	(Armstrong *et al*., 1994; Christensen *et al*., 1995; Tazi *et al*., 1993; Bleck *et al*., 2006)
IL-4	Promotes DC differentiation and maturation from monocytes in the presence of GM-CSF; increases LPS-induced release of IL-12 thus promotes Th1 immunity	(Hochrein *et al*., 2002)
IL-15	Promotes the differentiation of monocytes into DC; enhances DC maturation	(Bykovskaia *et al*., 1999)
TNFα, IL-1β, IL-6	Promote maturation of immature DC	(Gallucci *et al*., 1999)

Several studies have demonstrated the release by epithelial cells of chemokines capable of influencing DC migration, maturation and activation. The observations by Reibman *et al*. contribute a potentially important piece to this emerging jigsaw puzzle because they indicate how environmental exposures might influence functional maturation of airway mucosal DC and suggest a mechanism by which AEC might affect the dynamics of adjacent DC (Reibman *et al*., 2003). The study examined the ability of primary cultures of AEC to synthesize and secrete MIP-3/CCL20 (LARC, exodus-1). This CC chemokine is the unique ligand for the CCR6 chemokine receptor that is expressed on some immature DC, but not on CD14-positive DC precursors or mature DC. Human AEC were stimulated with pro-inflammatory cytokines, or small size-fractions of ambient particulate pollution. Each of these stimuli induced MIP-3/CCL20 gene and protein expression, suggesting a mechanism by which AEC may facilitate recruitment of DC subsets to the airway mucosa. The ultimate effects of such facilitated recruitment are likely to depend upon whether an individual is sensitized to aeroallergens, and the maturational stage of the adaptive immune system.

Studies utilizing in vitro co-culture of AEC and DC are being performed in several laboratories in order to examine the direct effects of epithelial cells on DC phenotype and function. For example, exposure of AEC/DC co-cultures to diesel exhaust particles induces phenotypic and functional maturation of immature DC and this effect is partly due to the actions of AEC-derived GM-CSF release (Bleck *et al*., 2006). Another group using the AEC/DC co-culture methodology showed that, in the context of bacterial stimulation, AEC were induced to express the chemokines CCL5 and CXCL10 that resulted in an increased

recruitment of monocyte-derived DC (MDDC) precursors into the epithelial space (Pichavant *et al*., 2006). AEC also promote the maturation of the MDDC precursors via a process that is mediated by ICAM-1/LFA-1 interactions and AEC-derived GM-CSF. Conditioning of DC by AEC also has downstream consequences leading to modification of T-cell cytokine output in an autologous DC/T-cell co-culture. Such in vitro studies should assist in elucidating the cellular and molecular mechanisms behind AEC regulation of DC and may, in turn, provide potential therapeutic targets for treatment of a number of respiratory diseases.

11.3 The pulmonary epithelium and lymphoid cells

Organized secondary lymphoid tissues are associated with all organ systems in the body and represent the primary sites for the initiation of T- and B-cell responses. As well as the pulmonary lymph nodes, additional, anatomically distinct collections of lymphoid cells may exist constitutively, or may be induced by infection or inflammation, to facilitate this process. It is clear that EC within the lung can interact with lymphocytes and, thereby, modulate their response to immune challenge. In addition, effector and memory lymphoid cells migrate out of these specialized lymphoid structures and into areas of inflamed or infected tissue. The following section outlines the organization of the lymphoid immune system in the lung, and the cross-talk that occurs between the pulmonary EC and the lymphocytes within the respiratory environment.

11.3.1 Organization of the lymphoid immune system in the lung

Bronchial-associated lymphoid tissue (BALT)

Bronchial-associated lymphoid tissue (BALT) refers to specially organized secondary lymphoid tissue lying adjacent to the airway lumen. It consists of highly organized follicles of B-cells surrounded closely by more diffuse lymphoid tissue known as the T-cell zone, and by follicle-associated epithelium (Sminia *et al*., 1989). The majority of cells within the BALT constitute B-cells that express surface IgA and IgM, with antibody-secreting plasma cells located only at the periphery (Otsuki *et al*., 1989). M cells are specialized EC that overlie the BALT and are derived from the basal stem cells of the respiratory epithelium. They have the ability to phagocytose and pinocytose molecules within their environment, and to deliver antigenic substrate to the underlying lymphoid tissue to generate immune responses (Neutra *et al*., 1996). Although they are well-characterized in the gastrointestinal tract mucosa, there are only a handful of studies identifying the presence of an M cell-like cell in the lung. Their potential to deliver antigen to the underlying lymphoid tissue suggests they may be crucial in developing an effective immune response in the respiratory tract (Tango *et al*., 2000; Teitelbaum *et al*., 1999).

The existence of BALT within the adult human respiratory tract has been a matter of debate. While animal models, particularly in rodents, have readily identified bona fide BALT present under normal conditions, there seems to be a relative absence of this organized tissue in the airway of healthy adult humans (Tschernig and Pabst, 2000). However, BALT has been identified in the lungs of human fetuses and is retained during adolescence, where it is thought to play a dominant role in the development of the pulmonary adaptive immune response (Hiller *et al*., 1998). In adults, however, the majority of the literature suggests that BALT is present only during periods of increased antigen exposure, such as chronic lung

disease (Tschernig and Pabst, 2000). However, one study looking at the lungs of healthy adult skiers showed that a high proportion of subjects displayed isolated aggregations of lymphoid cells (IALC) that may indeed correspond to a smaller version of BALT (Sue-Chu *et al*., 1998).

Isolated aggregations of lymphoid cells (IALC)

A recent study highlights a relatively novel variation of BALT that may be important in the mucosal lymphoid system of the lung under normal and diseased conditions (Elliot *et al*., 2004). IALC were defined as focal collections of greater than 50 lymphomononuclear cells with a density greater than 10 times that of the surrounding airway tissue. Like BALT, the lymphocytes in IALC were not randomly distributed but showed definite structural organization, with the majority of cells belonging to the B-cell population with large numbers of memory T-cells and small populations of antigen-presenting cells, macrophages and granulocytes. In contrast to BALT, there were no specialized EC present and, rather than being restricted to the upper airway, IALC appeared to be evenly distributed but were generally located away from the mucosal surface. Whilst IALC are present in healthy controls, numbers of these organized clusters were increased in the lungs of smokers and asthmatics, suggesting that, like BALT, elevated antigen exposure or tissue inflammation can drive their expansion. It was postulated that the role of IALC, both in healthy and diseased lungs, may be to facilitate local priming of B-cells and T-cells within the airway wall, rather than in the local draining lymph nodes.

11.4 The pulmonary epithelium and T-Cells

While priming of naive T-cells primarily occurs in the local lymph nodes of the lung, effector and memory T-cells, upon entry into the lung tissue, can be conditioned by the local environment, thus providing appropriate tissue-specific tuning of their responses. AEC produce a vast array of soluble mediators and surface-bound molecules that have the potential to influence T-cell function (reviewed in (Knight and Holgate, 2003)). They have been shown to play a central role in driving both the influx of certain T-cell subsets into the lung mucosa as well as the nature of the T-cell response once they are localized at the effector site. Thus, the regulation of T-cell responses by AEC has been shown to be critical in ensuring immune homeostasis of the lung tissue, as well as in initiating and maintaining defence against microbial invasion. Some of the mediators and surface molecules released by AEC that act on T-cells are shown in Figure 11.1.

Interleukin-6 (IL-6) is a pleiotropic cytokine that can have multiple effects on local T-cells. Signalling via the IL-6 receptor present on T-cells apparently suppresses the function of $CD4^+CD25^+$ T-regulatory cells in the lung (Hori *et al*., 2003). In addition, IL-6 skews a T-cell response to a Th2 direction under certain circumstances, even in the absence of a polarizing cytokine, whereas it impairs Th1 differentiation (Rincon *et al*., 1997). Thus, in allergic diseases of the respiratory tract, AEC-derived IL-6 can contribute to the pathogenesis of these conditions by creating a favourable environment for Th2 differentiation.

The arachidonic acid metabolite prostaglandin E2 (PGE_2) is constitutively expressed at low levels in AEC, but output of PGE_2 is significantly increased upon inflammatory insult. Associated with being a pro-inflammatory mediator in various disease states, PGE_2 has

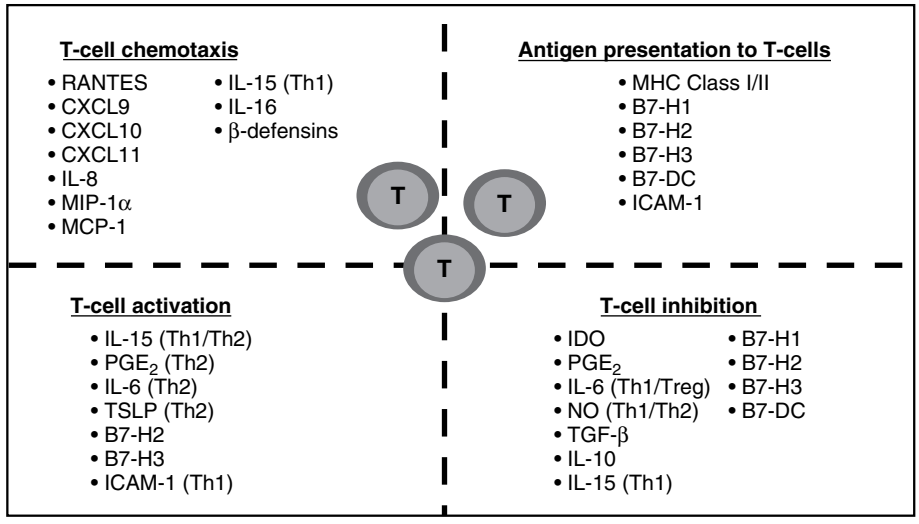


Figure 11.1 Pulmonary epithelial cells modulate T-cell function via their vast array of soluble mediators and surface-bound molecules

been shown to favour Th2 differentiation by inhibiting the production of IFN-γ in $CD4^+$ T-lymphocytes and, in the presence of IL-2, upregulating the production of IL-4 and IL-5 from these cells in vitro (Hilkens *et al*., 1995). However, inhalation of PGE_2 prevents early and late allergen-induced bronchoconstriction and decreases airway hyper-responsiveness and inflammation in asthma, indicating that, in fact, it acts in an immunosuppressive fashion (Gauvreau *et al*., 1999). T-cells are central to the late-phase response, and it has been shown that PGE_2 can limit this response by preventing the transendothelial migration of activated T-cells by affecting their intrinsic mobility and enhancing the barrier function of airway endothelial cells (Oppenheimer-Marks *et al*., 1994). In addition, despite the previously mentioned in vitro findings, experiments in animal models have revealed that there is a significant reduction in cells expressing IL-4 and IL-5 upon PGE_2 pre-treatment, suggesting that Th2 differentiation in vivo is inhibited (Martin *et al*., 2002).

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine produced primarily at the bronchial epithelial interface (Soumelis *et al*., 2002). This cytokine has been shown to drive a Th2-biased immune response, an effect mediated in part by the influence of TSLP on local dendritic cells (Watanabe *et al*., 2004; Wang *et al*., 2006). TSLP has also been shown to activate the release of a number of T-cell-attracting chemokines, both by the AEC themselves and by other neighbouring cells, including endothelial cells, neutrophils, macrophages, and mast cells. TARC/CCL17 and MDC/CCL22, levels of which are significantly increased in response to TSLP, selectively attract Th2-cells bearing their receptors. A study of asthmatic patients revealed higher levels of TSLP, and of both TARC and MDC, that were linked to the recruitment of large numbers of Th2-cells to the airway – a feature known to be important in the pathogenesis of this disease (Ying *et al*., 2005).

AEC have been defined as the most important source of nitric oxide (NO) in the airway (Donnelly and Barnes, 2002). Although some studies demonstrate that NO skews a T-cell

response towards a Th2 bias, this is probably an oversimplification, as NO can modulate both Th1 and Th2 subsets (Eriksson *et al*., 2005; Kolb and Kolb-Bachofen, 1998). It has been proposed that IFN-γ produced by Th1-cells feeds back on AEC, inducing nitric oxide synthase 2 (NOS-2) that, in turn, leads to increased NO release. The NO then induces a reversible growth arrest of both Th1 and Th2 cells by interfering with STAT5, a transcription factor involved in the IL-2R cascade. Thus, evidence now exists that, rather than being a disease-promoting mediator, AEC-derived NO has an important anti-inflammatory role in the lung, particularly in the context of allergic diseases such as asthma. The higher levels of NO observed in asthmatic lungs could thus reflect an active negative feedback mechanism (Lane *et al*., 2004).

Transforming growth factor-beta (TGFβ) and interleukin-10 (IL-10) are two molecules released by AEC that have potent immunomodulatory effects on T-cells. By blocking the transcription of IL-2, TGFβ inhibits proliferation of all naive T-cell subsets, although it is relatively ineffective on activated T-cells (Cerwenka and Swain, 1999; Cottrez and Groux, 2001). TGFβ is also a potent inhibitor of T-cell differentiation into Th subsets by interfering with the T-cell receptor (TCR) and co-stimulatory signalling pathways (Chen *et al*., 2003a). In addition, TGFβ regulates the differentiation of cytotoxic $CD8^+$ T-lymphocytes, as well as the induction of peripheral T regulatory cells (Tregs) (Chen *et al*., 2003b; Ranges *et al*., 1987). Thus, AEC expression of TGFβ primarily serves in the lungs to stringently regulate adaptive T-cell responses. IL-10, a known anti-inflammatory agent, has been shown to inhibit T-cell proliferation as well as their production of IL-2 and IFN-γ (Taga and Tosato, 1992). It is produced constitutively by normal human AEC, but its expression is down-regulated in some respiratory diseases, such as cystic fibrosis, where an aberrant regulation of T-cell responses contributes to the pathogenesis of this condition (Bonfield *et al*., 1995).

β-defensins are an important part of the lung's innate immune defence against microbial invasion. There are four human β-defensins, and all have been shown to be expressed either constitutively or inducibly by airway epithelia (Diamond *et al*., 1993). Despite their main antimicrobial role, β-defensins also communicate with the adaptive immune system. They have been shown to be chemotactic for cells expressing CCR6, such as memory T-cells (Yang *et al*., 1999). Thus, via the release of these peptides, AEC can recruit effector T-cells into the lung tissue to combat infection.

Interleukin-16 (IL-16) messenger RNA and protein is found in resting AEC and its expression is markedly increased in response to stimulation with a number of pro-inflammatory factors, including histamine, IL-1β and TNF-α (Arima *et al*., 1999). This cytokine induces the migratory response of $CD4^+$ T-cells, increases intracellular calcium and inositol 1,4,5-triphosphate levels, and induces the production of pro-inflammatory cytokines. High levels of IL-16 are found in the sputum of asthmatics, as well as in the lungs of patients with sarcoidosis, suggesting it has dual roles in both Th1 and Th2 respiratory disease.

Interleukin-15 (IL-15) is constitutively produced by AEC and is increased upon exposure to several pro-inflammatory stimuli (Ge *et al*., 2004). IL-15 favours the chemotaxis of Th1 cells, which bear an effective IL-15 receptor (Agostini *et al*., 1996). It can also synergize with IL-12 to promote the production of IFN-γ from T-cells and NK cells, which suggest that this cytokine drives a Th1-biased immune response (Seder, 1996). In this context, it has been reported that there is elevated expression of IL-15 in the lungs of patients with Th1-mediated inflammatory diseases compared to Th2-based conditions or healthy controls (Muro *et al*., 2001). However, IL-15 has been shown to promote the production of IL-5 by

Th lymphocytes, implying that it may play a role in allergic disease of the airway as well (Mori *et al*., 1996).

There are a number of other chemokines released by AEC, either in a constitutive or inducible fashion, that are important in the recruitment of T-cells into the airway. RANTES (regulated on activation, normal T-cell expressed and secreted), via a dual signalling pathway involving the CCR5 receptor, promotes the chemotaxis of T-cells. It also induces their proliferation, and stimulates expression of a number of cytokines, including IL-2 and IL-5, and cytokine receptors (Bacon *et al*., 1995). ICAM-1 cross-linking on the surface of AEC has been shown to up-regulate the production of RANTES (Krunkosky and Jarrett, 2006). CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), are ligands for CXCR3 that are all expressed by activated AEC (Sauty *et al*., 1999). The interaction of these molecules with their receptor was shown to significantly diminish T-cell recruitment and lung pathology in a model of idiopathic pneumonia syndrome (Hildebrandt *et al*., 2004). Ciliated human AEC have been demonstrated to express IL-8 at baseline (Devalia *et al*., 1993), and levels are increased in response to several stimuli, including viruses and diesel exhaust particles. Although a major chemotactic factor for neutrophils, this molecule can also recruit T-cells into inflamed areas (Baggiolini *et al*., 1994). Macrophage inflammatory peptide 1α (MIP-1α) is constitutively present in the airway of both normal subjects, suggesting that it may regulate T-cell trafficking under physiologic conditions (Holgate *et al*., 1997). Finally, monocyte chemoattractant protein (MCP)-1/CCL2 is released by AEC upon stimulation and attracts memory T-cells (Daly and Rollins, 2003). In murine models this molecule has been shown to promote Th2-mediated immunity, although there is no evidence to date that this applies in humans (Matsukawa *et al*., 2000).

Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting, tryptophan-catabolizing enzyme that is expressed in a variety of cells found in the respiratory environment, including AEC upon stimulation with IFN-γ (Zegarra-Moran *et al*., 2004). The expression of IDO is further enhanced by ligand-induced activation of various toll-like receptors (Babcock and Carlin, 2000). The enzymatic actions of IDO lead to depletion of tryptophan in the local environment, resulting in the inhibition of T-cell proliferation (Mellor *et al*., 2002). In addition, IDO promotes the generation of a number of toxic tryptophan metabolites that can induce Th cell death (Frumento *et al*., 2002). Thus, in the event of inflammation, AEC can contribute to the regulation of the adaptive immune response in order to limit tissue damage.

11.4.1 Antigen presentation by airway epithelial cells

Dendritic cells (DC) have been established as the major class of antigen-presenting cell (APC) in the airway, capable of taking up, processing and presenting antigen to $CD4^+$ T-lymphocytes (Banchereau and Steinman, 1998). To a lesser degree, alveolar macrophages also participate in the presentation of inhaled antigen, although they have also been shown to down-regulate the APC properties of local DC in order to suppress immune responses (Holt *et al*., 1993). The first evidence of EC possessing antigen presentation capabilities arose from studies in the rat showing that intestinal EC could present antigen to lymph node T-cells and stimulate their proliferation (Bland and Warren, 1986). Following these observations in animals, human gastro-intestinal tract epithelial cells were demonstrated to possess similar accessory cell function in in vitro studies using EC isolated from surgically resected gut mucosa (Mayer and Shlien, 1987). More recent studies, focusing on AEC in both animal and human models have demonstrated similar capacity for antigen presentation

that, like their professional APC counterparts, is antigen-specific and can elicit an efficient response in responder T-cells (Suda *et al*., 1995).

AEC can express several molecules involved in antigen presentation, including major histocompatibility (MHC) class I and II, and a number of co-stimulatory molecules, although there are conflicting reports about constitutive levels of these markers. Initial studies revealed that both class I (HLA-A,B,C) and class II (HLA-DR) antigens were uniformly and strongly expressed on the bronchiolar and alveolar epithelium (Glanville *et al*., 1989). In contrast, another group demonstrated high levels of class I but undetectable levels of class II on both primary AEC and cell lines (Papi *et al*., 2000). A third study showed that freshly isolated alveolar EC constitutively express high levels of class II molecules, whereas cultured small airway EC did not show detectable levels of this marker (Cunningham *et al*., 1997). These inconsistencies may be explained by the presence of occult inflammation or injury at the time of sampling, but the critical role of MHC molecules in antigen presentation by EC is generally agreed upon. One group showed that both freshly isolated and short-term cultured AEC were able to stimulate allogeneic T-lymphocytes in a mixed lymphocyte reaction (MLR), and this could be completely inhibited by the addition of an anti-class II monoclonal antibody at the onset of culture (Kalb *et al*., 1991). In addition, the alveolar EC line A549, which does not express MHC class II molecules, cannot stimulate T-cell proliferation (Paine *et al*., 1992).

The antigen uptake and presentation capacities of AEC, like their professional APC counterparts, are responsive to the local cytokine microenvironment. It has been demonstrated in several studies that IFN-γ and granulocyte–macrophage colony stimulating factor (GM-CSF) can positively affect the magnitude and kinetics of this process in local AEC, and several cell types in intimate contact with AEC can produce these two cytokines. IFN-γ- and GM-CSF-treated AEC exhibit increased antigen uptake and processing, which occurs at an earlier time point than untreated cells (Oei *et al*., 2004). The presence of IFN-γ was shown to enhance surface expression of MHC II and FcγR on AEC, whereas when this cytokine was absent, there was a lack of persistent expression of MHC II on the cell surface (Salik *et al*., 1999; Rossi *et al*., 1990).

Optimal activation of T-cells requires not only engagement of the TCR receptor, but also co-stimulation by a number of different molecules expressed by APCs, which, when absent, results in T-cell unresponsiveness or anergy (Mueller *et al*., 1989). In particular, the B7-family of ligands has a central role in T-cell co-stimulation, with B7-1 (CD80) and B7-2 (CD86) shown to be central to the activation of T-cells by DC through ligation of the CD28 receptor on T-cells (Bugeon and Dallman, 2000; Chambers and Allison, 1997). Studies of these B7-1 and B7-2 on airway epithelial cells have found an absence of their constitutive expression in both freshly isolated and immortalized cells (Cunningham *et al*., 1997; Kurosawa *et al*., 2003). In addition, unlike MHC Class II expression, the levels of these co-stimulatory molecules on AEC were unchanged in response to IFN-γ and GM-CSF, as well as to a number of other immunomodulatory cytokines examined, including IL-10, IL-5 and IL-4 (Oei *et al*., 2004). More recent identification of homologues of B7-1 and B7-2 on AEC suggest that other co-stimulatory molecules may play a role in the accessory function of these cells. One report identified the constitutive expression of B7-H1 (programmed death ligand 1 (PDL-1)), B7-H2 (inducible co-stimulatory molecule ligand (ICOS-L)), B7-H3 and B7-DC (programmed death ligand 2 (PDL-2)), on both primary and immortalized AEC. B7-H1 and B7-DC, ligands for PD-1, which is an inhibitory receptor on T-cells, were up-regulated in response to stimulation with IFN-γ and TNF-α, and were shown to inhibit T-cell cytokine responses in a AEC/T-cell co-culture. In contrast B7-H2

and B7-H3, whose expression levels were considerably higher than those of B7-H1 and B7-DC, can operate as activating co-stimulatory molecules in the same system, although they have also been reported to exhibit some inhibitory activity under certain conditions (Kim *et al*., 2005; Suh *et al*., 2003; Subudhi *et al*., 2005). In light of this data, it is anticipated that AEC are intimately involved in the regulation of function and survival of T-cells within the airway by virtue of their expression of these co-stimulatory molecules. In some circumstances, AEC enhance T-cell activation, while in other situations AEC can inhibit T-cell responses.

Human AEC constitutively express intercellular adhesion molecule 1 (ICAM-1) (Papi *et al*., 2000). Although this molecule is classically known as an adhesion receptor, it also possesses co-stimulatory properties. Whilst the B7 family of molecules is critical for MHC II-restricted antigen presentation, APC presenting MHC I-restricted antigens often do so in the absence of B7 co-stimulation. It is under these conditions that ICAM-1 serves as a source of co-stimulation to CD8+ T-cells through ligation of LFA-1 (Gaglia *et al*., 2000). In addition, binding of LFA-1 and ICAM-1 has been shown to preferentially drive Th1 responses, a mechanism that may occur during viral infections (Smits *et al*., 2002).

Antigen uptake and trafficking processes in AEC have been examined in detail. Exogenous antigen is taken up by AEC in a non-receptor mediated fashion, and follows a class II endocytic pathway, as evidenced by co-localization of labelled antigen with various early and late endosomal and lysosomal markers at different time-points (Salik *et al*., 1999). In addition, a second group demonstrated the presence in freshly isolated AEC of a number of cathepsins that are important for antigen processing (Oei *et al*., 2004). The same group examined the expression of FcγR on the surface of these cells and found that it was upregulated by IFN-γ and could serve to act as a channel for uptake of immunoglobulin G (IgG) immune complexes by AEC.

The phenotype of the T-cells responding to antigen presentation by AEC has also been investigated. One group showed that AEC induced the proliferation of both $CD4^+$ and $CD8^+$ T-cells bearing the memory phenotype ($CD45RO^+CCR7^+$). In contrast, DC were able to stimulate both naive ($CD45RA^+CCR7^-$) and memory T-cells (Oei *et al*., 2004). The authors postulate that AEC may be able to provide the necessary signals for reactivation of memory T-cells in the peripheral tissue, whereas DC may play a larger role in primary immune responses and in systemic immune activation. In addition, in contrast to intestinal EC where induction of a T-cell response is limited to the $CD8^+$ subset, AEC activate both $CD4^+$ and $CD8^+$ T-cells, which may reflect differences in the requirements of these cells to serve as APC in their local mucosal environments (Kalb *et al*., 1997)

Viral infection of the respiratory tract has been demonstrated as the major cause of asthma exacerbations in children and adults (Chapter 12). Infection of AEC by viruses has been shown to modulate their expression of MHC and co-stimulatory molecules and, accordingly, may play a role in the cellular and molecular mechanisms of viral-induced asthma. Rhinovirus, which utilizes the intercellular adhesion molecule (ICAM)-1 as its major receptor, selectively increases the expression of Class I but not Class II molecules on immortalized lines and primary AEC upon infection (Papi *et al*., 2000). In addition, this study also found that the expression of classic co-stimulatory molecules B7-1 and B7-2 is induced upon respiratory tract viral infection. Respiratory syncytial virus (RSV), another common cause of respiratory tract infection, has been shown to up-regulate the expression of other B7-family co-stimulatory molecules B7-H1, B7-H2, B7-H3 and B7-DC (Stanciu *et al*., 2006). As these molecules all possess T-cell inhibitory function, it is hypothesized that

RSV can restrict effector T-cell activation upon infection of AEC, thereby evading detection and elimination by the host immune system.

Therapeutic drugs target various aspects of the accessory APC function to down-regulate immune responses in the lung. For example, sodium nedocromil, used widely in the treatment of asthma, suppresses the expression of HLA-DR antigen and ICAM-1 molecule expression on airway epithelial cells (Sacco *et al*., 1999). Fluticasone, a potent glucocorticoid, has been shown to inhibit the induction of co-stimulatory molecules B7-H1 and B7-DC in BEAS-2B cells (Kim *et al*., 2005). In addition, certain adjuvants in viral vaccines preclude the antigen presentation of virus to cytotoxic $CD8^+$ T-cells and thus enable them to avoid being killed by virus-specific CTL, a favourable outcome in mucosal vaccination strategies (Rimmelzwaan *et al*., 2004).

11.5 The pulmonary epithelium and gamma/delta T-cells

Although in relatively low numbers in the circulation, γδ T-cells constitute a major proportion of the lymphocytes found within the respiratory epithelium (Augustin *et al*., 1989). These cells arise from the same precursors in the thymus as their αβ counterparts and home to epithelia surfaces under the influence of various soluble factors and surface markers (Sim *et al*., 1994). While their TCR repertoire is very limited compared to αβ T-cells, it is thought that γδ T-cells may recognize tissue-specific antigens and play a vital role in maintaining lung homeostasis (Havran *et al*., 2005).

By virtue of their intimate contact with surrounding EC, γδ T-cells are vital in maintaining the homeostasis of these cells in mucosal sites (as reviewed in Komori *et al*., 2006). A recent study has revealed that stressed or damaged airway epithelium expresses ligands for the NKG2D-activating receptor found on γδ T-cells. Triggering of the γδ T-cells within the mucosal epithelium can lead to the efficient removal of the compromised cell, thus preventing the chronic activation of the epithelium and avoiding a prolonged immune response. In the absence of γδ T-cells, EC injury is more severe and there is increased risk for development of chronic airway disease (Borchers *et al*., 2006). It is thought that γδ T-cells also promote the production of specific growth factors and other inflammatory mediators by epithelial cells to accelerate the repair of damaged tissue. Recent evidence from the skin shows a direct role for γδ T-cells in inducing hyaluronan production by EC that in turn recruits macrophages to the site of damage. It has been suggested that a similar scenario operates in the lungs (Jameson *et al*., 2005).

11.6 The pulmonary epithelium, B-cells and IgA production

Secretory IgA (sIgA) is a crucial component of the immunological barrier factors of mucous membranes. The production of sIgA is a multi-step process involving the heavy chain class-switching of activated B-cells, and the subsequent differentiation of these IgA-dedicated B-cells to antibody-secreting plasma cells. This process occurs locally within the mucosa and is exquisitely sensitive to the local cytokine microenvironment. IgA-producing immunocytes represent the dominant mature plasma cell in mucosal surfaces and, due to co-operation between the mucosal lymphoid tissue and the surrounding epithelium, a continuous production of polymeric sIgA is maintained (Pilette *et al*., 2001).

Polymeric sIgA is the most abundant immunoglobulin in the secretions of the upper respiratory tract and forms part of the first line of immune defense in the lung (Burnett, 1986). This specialized isotype exerts its protective effects in a number of ways, primarily by the formation of complexes with micro-organisms that prevents binding to mucosal surfaces and accelerates their clearance by other effector cells of the immune system (Lamm, 1997). In addition, ligation of IgA receptors can generate a wide range of biological responses in other effector cells of the immune system, such as antibody-dependent cellular cytotoxicity (ADCC), release of cytokines and superoxide generation (as reviewed in Monteiro and Van De Winkel, 2003).

The pleiotropic cytokine transforming growth factor beta-1 (TGFβ1) has been ascribed a number of immunosuppressive and anti-inflammatory roles, including its critical function in the induction of IgA responses in vivo. TGFβ induces IgA switching in B-cells by activating transcription through the Cα locus, an effect that is apparent after only a short exposure of uncommitted B-cells to TGFβ (van Vlasselaer *et al*., 1992). Studies of TGFβ1-deficient and TGFβ 1R-deficient have shown that these animals exhibit a partial IgA deficiency (Cazac and Roes, 2000; van Ginkel *et al*., 1999). Likewise in studies of humans, IgA deficiency is associated with reduced serum levels of TGFβ (Muller *et al*., 1995), whereas patients displaying IgA-mediated nephropathies exhibit increased levels of TGFβ mRNA (Lai *et al*., 1994). While TGFβ production by AEC is significantly increased in epithelial remodelling and repair (Howat *et al*., 2002), there is constitutive expression of TGF that supports homeostatic paracrine interactions between normal cells within the epithelial–mesenchymal unit (Wang *et al*., 1996). In addition, ligation of CD40 on B-cells by CD40 ligand expressed on the AEC of normal subjects, triggers switching to IgA1 and IgA2 via induction of endogenous TGFβ (Vignola *et al*., 2001; Zan *et al*., 1998). Hence, AEC support IgA production under normal conditions, and in situations of inflammation or injury, this is enhanced by virtue of increased TGFβ expression.

In addition to TGFβ, AEC produce a number of other cytokines that promote terminal differentiation of mature IgA^+-B-cells towards IgA-producing plasma cells. Although controversial, interleukin-5 (IL-5) has been reported to be produced constitutively by both primary and transformed AEC, and to be up-regulated under inflammatory conditions (Salvi *et al*., 1999). It has been shown to play a vital role in the homeostatic proliferation and survival of mature B-1 cells. This is the subset of B-cells that serves as an important source of IgA-producing plasma cells at mucosal sites that can be activated independently of T-cell help (Moon *et al*., 2004). IL-5 has a synergistic effect with TGFβ. While the growth factor targets the isotype switch process of activated B-cells to IgA, IL-5 acts downstream of this process, driving the differentiation of the IgA+ effector plasma cells (Sonoda *et al*., 1989). Similarly to IL-5, IL-6 has been shown to drive the process of transition of IgA-committed B-cells to IgA-producing plasma cells. In contrast to the former, IL-6 drives expansion of the B-2 subset of committed B-cells (Beagley *et al*., 1989).

Human IL-10 is known to induce the synthesis of IgG1, IgG3 and IgA in anti-CD40 Ab-activated naive B-cells (Hummelshoj *et al*., 2006). If these B cells are cultured in the presence of TGFβ, the production of IgA is increased further, suggesting a synergistic effect of these two cytokines. IL-10 has also been shown to upregulate the expression of IL-2 receptors on the surface of B-cells (Fluckiger *et al*., 1993). In conjunction, AEC also release IL-2 that operates on B cells to promote their initial activation and expansion prior to isotype switching and downstream maturation.

AEC may also benefit directly from sIgA production. EC act as conduits to transport IgA produced within the lamina propria lining the airway to the lumen where it can exert its effector function. Transcytosis of sIgA from the basal to apical surfaces of EC occurs via a specialized receptor produced by the epithelium known as the secretory component (SC). This receptor functions in two ways: firstly, to bind the IgA monomers together; and secondly, to prevent proteolytic destruction of the antibody once inside the airway lumen (Lindh, 1975). Thus, while successful transport and survival of secretory IgA in the airway is intimately dependent on the existence of the AEC population, the epithelial cells can exploit the IgA for their own protection.

11.7 Conclusions

The evidence outlined in this chapter collectively demonstrates that AEC are not a simple barrier but, in fact, play an active role in the immune response. It is clear that by virtue of a vast array of soluble mediators and surface molecules, they not only can participate in the initiation of an adaptive immune response, but also can influence the nature and outcome of that response.

References

Agostini, C., Trentin, L., Facco, M., Sancetta, R., Cerutti, A., Tassinari, C., Cimarosto, L., Adami, F., Cipriani, A., Zambello, R. & Semenzato, G. (1996) Role of IL-15, IL-2, and their receptors in the development of T cell alveolitis in pulmonary sarcoidosis, *Journal of Immunology*, **157**, 910–918.

Akbari, O., DeKruyff, R. H. & Umetsu, D. T. (2001) Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen, *Nature Immunology*, **2**, 725–731.

Arima, M., Plitt, J., Stellato, C., Bickel, C., Motojima, S., Makino, S., Fukuda, T. & Schleimer, R. P. (1999) Expression of interleukin-16 by human epithelial cells. Inhibition by dexamethasone, *American Journal of Respiratory Cell and Molecular Biology*, **21**, 684–692.

Armstrong, L. R., Christensen, P. J., Paine, R., 3rd, Chen, G. H., McDonald, R. A., Lim, T. K. & Toews, G. B. (1994) Regulation of the immunostimulatory activity of rat pulmonary interstitial dendritic cells by cell–cell interactions and cytokines, *American Journal of Respiratory Cell and Molecular Biology*, **11**, 682–691.

Augustin, A., Kubo, R. T. & Sim, G. K. (1989) Resident pulmonary lymphocytes expressing the gamma/delta T-cell receptor, *Nature*, **340**, 239–241.

Babcock, T. A. & Carlin, J. M. (2000) Transcriptional activation of indoleamine dioxygenase by interleukin 1 and tumor necrosis factor alpha in interferon-treated epithelial cells, *Cytokine*, **12**, 588–594.

Bacon, K. B., Premack, B. A., Gardner, P. & Schall, T. J. (1995) Activation of dual T cell signaling pathways by the chemokine RANTES, *Science*, **269**, 1727–1730.

Baggiolini, M., Dewald, B. & Moser, B. (1994) Interleukin-8 and related chemotactic cytokines – CXC and CC chemokines, *Advances in Immunology*, **55**, 97–179.

Banchereau, J. & Steinman, R. M. (1998) Dendritic cells and the control of immunity, *Nature*, **392**, 245–252.

Beagley, K. W., Eldridge, J. H., Lee, F., Kiyono, H., Everson, M. P., Koopman, W. J., Hirano, T., Kishimoto, T. & McGhee, J. R. (1989) Interleukins and IgA synthesis. Human and murine interleukin

6 induce high rate IgA secretion in IgA-committed B cells, *Journal of Experimental Medicine*, **169**, 2133–2148.

Biragyn, A., Ruffini, P. A., Leifer, C. A., Klyushnenkova, E., Shakhov, A., Chertov, O., Shirakawa, A. K., Farber, J. M., Segal, D. M., Oppenheim, J. J. & Kwak, L. W. (2002) Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2, *Science*, **298**, 1025–9.

Bland, P. W. & Warren, L. G. (1986) Antigen presentation by epithelial cells of the rat small intestine. I. Kinetics, antigen specificity and blocking by anti-Ia antisera, *Immunology*, **58**, 1–7.

Bleck, B., Tse, D. B., Jaspers, I., Curotto de Lafaille, M. A. & Reibman, J. (2006) Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation, *Journal of Immunology*, **176**, 7431–7437.

Bonfield, T. L., Konstan, M. W., Burfeind, P., Panuska, J. R., Hilliard, J. B. & Berger, M. (1995) Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis, *American Journal of Respiratory Cell and Molecular Biology*, **13**, 257–261.

Borchers, M. T., Harris, N. L., Wesselkamper, S. C., Vitucci, M. & Cosman, D. (2006) NKG2D ligands are expressed on stressed human airway epithelial cells, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, **291**, L222–L231.

Borkowski, T. A., Letterio, J. J., Farr, A. G. & Udey, M. C. (1996) A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells, *Journal of Experimental Medicine*, **184**, 2417–2422.

Bugeon, L. & Dallman, M. J. (2000) Costimulation of T cells, *American Journal of Respiratory and Critical Care Medicine*, **162**, S164–S168.

Burnett, D. (1986) Immunoglobulins in the lung, *Thorax*, **41**, 337–344.

Bykovskaia, S. N., Buffo, M., Zhang, H., Bunker, M., Levitt, M. L., Agha, M., Marks, S., Evans, C., Ellis, P., Shurin, M. R. & Shogan, J. (1999) The generation of human dendritic and NK cells from hemopoietic progenitors induced by interleukin-15, *Journal of Leukocyte Biology*, **66**, 659–666.

Carayol, N., Campbell, A., Vachier, I., Mainprice, B., Bousquet, J., Godard, P. & Chanez, P. (2002) Modulation of cadherin and catenins expression by tumor necrosis factor-alpha and dexamethasone in human bronchial epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **26**, 341–347.

Cazac, B. B. & Roes, J. (2000) TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo, *Immunity*, **13**, 443–451.

Cerwenka, A. & Swain, S. L. (1999) TGF-beta1: immunosuppressant and viability factor for T lymphocytes, *Microbes and Infection*, **1**, 1291–1296.

Chambers, C. A. & Allison, J. P. (1997) Co-stimulation in T cell responses, *Current Opinion in Immunology*, **9**, 396–404.

Chen, C. H., Seguin-Devaux, C., Burke, N. A., Oriss, T. B., Watkins, S. C., Clipstone, N. & Ray, A. (2003a) Transforming growth factor beta blocks Tec kinase phosphorylation, Ca2+ influx, and NFATc translocation causing inhibition of T cell differentiation, *Journal of Experimental Medicine*, **197**, 1689–1699.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G. & Wahl, S. M. (2003b) Conversion of peripheral CD4 + CD25− naive T cells to $CD4^{+}CD25^{+}$ regulatory T cells by TGF-beta induction of transcription factor Foxp3, *Journal of Experimental Medicine*, **198**, 1875–1886.

Christensen, P. J., Armstrong, L. R., Fak, J. J., Chen, G. H., McDonald, R. A., Toews, G. B. & Paine, R., 3rd (1995) Regulation of rat pulmonary dendritic cell immunostimulatory activity by alveolar epithelial cell-derived granulocyte macrophage colony-stimulating factor, *American Journal of Respiratory Cell and Molecular Biology*, **13**, 426–433.

Cottrez, F. & Groux, H. (2001) Regulation of TGF-beta response during T cell activation is modulated by IL-10, *Journal of Immunology*, **167**, 773–778.

Cunningham, A. C., Zhang, J. G., Moy, J. V., Ali, S. & Kirby, J. A. (1997) A comparison of the antigen-presenting capabilities of class II MHC-expressing human lung epithelial and endothelial cells, *Immunology*, **91**, 458–463.

Daly, C. & Rollins, B. J. (2003) Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies, *Microcirculation*, **10**, 247–57.

Devalia, J. L., Campbell, A. M., Sapsford, R. J., Rusznak, C., Quint, D., Godard, P., Bousquet, J. & Davies, R. J. (1993) Effect of nitrogen dioxide on synthesis of inflammatory cytokines expressed by human bronchial epithelial cells in vitro, *American Journal of Respiratory Cell and Molecular Biology*, **9**, 271–278.

Diamond, G., Jones, D. E. & Bevins, C. L. (1993) Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene, *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 4596–4600.

Donnelly, L. E. & Barnes, P. J. (2002) Expression and regulation of inducible nitric oxide synthase from human primary airway epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **26**, 144–151.

Elliot, J. G., Jensen, C. M., Mutavdzic, S., Lamb, J. P., Carroll, N. G. & James, A. L. (2004) Aggregations of lymphoid cells in the airways of nonsmokers, smokers, and subjects with asthma, *American Journal of Respiratory and Critical Care Medicine*, **169**, 712–718.

Eriksson, U., Egermann, U., Bihl, M. P., Gambazzi, F., Tamm, M., Holt, P. G. & Bingisser, R. M. (2005) Human bronchial epithelium controls TH2 responses by TH1-induced, nitric oxide-mediated STAT5 dephosphorylation: implications for the pathogenesis of asthma, *Journal of Immunology*, **175**, 2715–2720.

Fluckiger, A. C., Garrone, P., Durand, I., Galizzi, J. P. & Banchereau, J. (1993) Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes, *Journal of Experimental Medicine*, **178**, 1473–1481.

Frumento, G., Rotondo, R., Tonetti, M., Damonte, G., Benatti, U. & Ferrara, G. B. (2002) Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase, *Journal of Experimental Medicine*, **196**, 459–468.

Fujimoto, K., Imaizumi, T., Yoshida, H., Takanashi, S., Okumura, K. & Satoh, K. (2001) Interferon-gamma stimulates fractalkine expression in human bronchial epithelial cells and regulates mononuclear cell adherence, *American Journal of Respiratory Cell and Molecular Biology*, **25**, 233–238.

Fujita, H., Asahina, A. & Tamaki, K. (2006) Ligation of E-cadherin molecules on murine resident Langerhans cells inhibits their maturation and chemokine production, *Journal of Dermatological Science*, **43**, 152–155.

Gaglia, J. L., Greenfield, E. A., Mattoo, A., Sharpe, A. H., Freeman, G. J. & Kuchroo, V. K. (2000) Intercellular adhesion molecule 1 is critical for activation of CD28-deficient T cells, *Journal of Immunology*, **165**, 6091–6098.

Gallucci, S., Lolkema, M. & Matzinger, P. (1999) Natural adjuvants: endogenous activators of dendritic cells, *Nature Medicine*, **5**, 1249–1255.

Gauvreau, G. M., Watson, R. M. & O'Byrne, P. M. (1999) Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation, *American Journal of Respiratory and Critical Care Medicine*, **159**, 31–36.

Ge, N., Nishioka, Y., Nakamura, Y., Okano, Y., Yoneda, K., Ogawa, H., Sugita, A., Yanagawa, H. & Sone, S. (2004) Synthesis and secretion of interleukin-15 by freshly isolated human bronchial epithelial cells, *International Archives of Allergy and Immunology*, **135**, 235–242.

Geissmann, F., Prost, C., Monnet, J. P., Dy, M., Brousse, N. & Hermine, O. (1998) Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells, *Journal of Experimental Medicine*, **187**, 961–966.

Geissmann, F., Revy, P., Regnault, A., Lepelletier, Y., Dy, M., Brousse, N., Amigorena, S., Hermine, O. & Durandy, A. (1999) TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells, *Journal of Immunology*, **162**, 4567–4575.

Glanville, A. R., Tazelaar, H. D., Theodore, J., Imoto, E., Rouse, R. V., Baldwin, J. C. & Robin, E. D. (1989) The distribution of MHC class I and II antigens on bronchial epithelium, *American Review of Respiratory Disease*, **139**, 330–334.

Gosset, P., Bureau, F., Angeli, V., Pichavant, M., Faveeuw, C., Tonnel, A. B. & Trottein, F. (2003) Prostaglandin D2 affects the maturation of human monocyte-derived dendritic cells: consequence on the polarization of naive Th cells, *Journal of Immunology*, **170**, 4943–4952.

Greene, C. M., McElvaney, N. G., O'Neill, S. J. & Taggart, C. C. (2004) Secretory leucoprotease inhibitor impairs Toll-like receptor 2- and 4-mediated responses in monocytic cells, *Infection and Immunity*, **72**, 3684–3687.

Havran, W. L., Jameson, J. M. & Witherden, D. A. (2005) Epithelial cells and their neighbors. III. Interactions between intraepithelial lymphocytes and neighboring epithelial cells, *American Journal of Physiology – Gastointestinal and Liver Physiology*, **289**, G627–G630.

Hayday, A. & Viney, J. L. (2000) The ins and outs of body surface immunology, *Science*, **290**, 97–100.

Henriksen, P. A., Hitt, M., Xing, Z., Wang, J., Haslett, C., Riemersma, R. A., Webb, D. J., Kotelevtsev, Y. V. & Sallenave, J. M. (2004) Adenoviral gene delivery of elafin and secretory leukocyte protease inhibitor attenuates NF-kappa B-dependent inflammatory responses of human endothelial cells and macrophages to atherogenic stimuli, *Journal of Immunology*, **172**, 4535–4544.

Hildebrandt, G. C., Corrion, L. A., Olkiewicz, K. M., Lu, B., Lowler, K., Duffner, U. A., Moore, B. B., Kuziel, W. A., Liu, C. & Cooke, K. R. (2004) Blockade of CXCR3 receptor:ligand interactions reduces leukocyte recruitment to the lung and the severity of experimental idiopathic pneumonia syndrome, *Journal of Immunology*, **173**, 2050–2059.

Hilkens, C. M., Vermeulen, H., van Neerven, R. J., Snijdewint, F. G., Wierenga, E. A. & Kapsenberg, M. L. (1995) Differential modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2, *European Journal of Immunology*, **25**, 59–63.

Hiller, A. S., Tschernig, T., Kleemann, W. J. & Pabst, R. (1998) Bronchus-associated lymphoid tissue (BALT) and larynx-associated lymphoid tissue (LALT) are found at different frequencies in children, adolescents and adults, *Scandinavian Journal of Immunology*, **47**, 159–162.

Hochrein, H., O'Keeffe, M. & Wagner, H. (2002) Human and mouse plasmacytoid dendritic cells, *Human Immunology*, **63**, 1103–1110.

Holgate, S. T., Bodey, K. S., Janezic, A., Frew, A. J., Kaplan, A. P. & Teran, L. M. (1997) Release of RANTES, MIP-1 alpha, and MCP-1 into asthmatic airways following endobronchial allergen challenge, *American Journal of Respiratory and Critical Care Medicine*, **156**, 1377–1383.

Holgate, S. T., Lackie, P., Wilson, S., Roche, W. & Davies, D. (2000) Bronchial epithelium as a key regulator of airway allergen sensitization and remodeling in asthma, *American Journal of Respiratory and Critical Care Medicine*, **162**, S113–S117.

Holt, P. G., Oliver, J., Bilyk, N., McMenamin, C., McMenamin, P. G., Kraal, G. & Thepen, T. (1993) Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages, *Journal of Experimental Medicine*, **177**, 397–407.

Holt, P. G. & Upham, J. W. (2004) The role of dendritic cells in asthma, *Current Opinion in Allergy and Clinical Immunology*, **4**, 39–44.

Hori, S., Nomura, T. & Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3, *Science*, **299**, 1057–1061.

Howat, W. J., Holgate, S. T. & Lackie, P. M. (2002) TGF-beta isoform release and activation during in vitro bronchial epithelial wound repair, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **282**, L115–L123.

Huh, J. C., Strickland, D. H., Jahnsen, F. L., Turner, D. J., Thomas, J. A., Napoli, S., Tobagus, I., Stumbles, P. A., Sly, P. D. & Holt, P. G. (2003) Bidirectional interactions between antigen-bearing

respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma, *Journal of Experimental Medicine*, **198**, 19–30.

Hummelshoj, L., Ryder, L. P. & Poulsen, L. K. (2006) The role of the interleukin-10 subfamily members in immunoglobulin production by human B cells, *Scandinavian Journal of Immunology*, **64**, 40–47.

Jahnsen, F. L., Moloney, E. D., Hogan, T., Upham, J. W., Burke, C. M. & Holt, P. G. (2001) Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge, *Thorax*, **56**, 823–826.

Jameson, J. M., Cauvi, G., Sharp, L. L., Witherden, D. A. & Havran, W. L. (2005) Gammadelta T cell-induced hyaluronan production by epithelial cells regulates inflammation, *Journal of Experimental Medicine*, **201**, 1269–1279.

Kalb, T. H., Chuang, M. T., Marom, Z. & Mayer, L. (1991) Evidence for accessory cell function by class II MHC antigen-expressing airway epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **4**, 320–329.

Kalb, T. H., Yio, X. Y. & Mayer, L. (1997) Human airway epithelial cells stimulate T-lymphocyte lck and fyn tyrosine kinase, *American Journal of Respiratory Cell and Molecular Biology*, **17**, 561–570.

Kalinski, P., Schuitemaker, J. H., Hilkens, C. M. & Kapsenberg, M. L. (1998) Prostaglandin E2 induces the final maturation of IL-12-deficient $CD1a^{+}CD83^{+}$ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation, *Journal of Immunology*, **161**, 2804–2809.

Kim, J., Myers, A. C., Chen, L., Pardoll, D. M., Truong-Tran, Q. A., Lane, A. P., McDyer, J. F., Fortuno, L. & Schleimer, R. P. (2005) Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **33**, 280–289.

Knight, D. A. & Holgate, S. T. (2003) The airway epithelium: structural and functional properties in health and disease, *Respirology*, **8**, 432–446.

Kolb, H. & Kolb-Bachofen, V. (1998) Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? *Immunology Today*, **19**, 556–561.

Komori, H. K., Meehan, T. F. & Havran, W. L. (2006) Epithelial and mucosal gammadelta T cells, *Current Opinion in Immunology*, **18**, 534–538.

Krunkosky, T. M. & Jarrett, C. L. (2006) Selective regulation of MAP kinases and chemokine expression after ligation of ICAM-1 on human airway epithelial cells, *Respiratory Research*, **7**, 12.

Kuppner, M. C., Gastpar, R., Gelwer, S., Nossner, E., Ochmann, O., Scharner, A. & Issels, R. D. (2001) The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors, *European Journal of Immunology*, **31**, 1602–1609.

Kurosawa, S., Myers, A. C., Chen, L., Wang, S., Ni, J., Plitt, J. R., Heller, N. M., Bochner, B. S. & Schleimer, R. P. (2003) Expression of the costimulatory molecule B7-H2 (inducible costimulator ligand) by human airway epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **28**, 563–573.

Lai, K. N., Ho, R. T., Leung, J. C., Lai, F. M. & Li, P. K. (1994) Increased mRNA encoding for transforming factor-beta in CD4+ cells from patients with IgA nephropathy, *Kidney International*, **46**, 862–868.

Lambrecht, B. N., De Veerman, M., Coyle, A. J., Gutierrez-Ramos, J. C., Thielemans, K. & Pauwels, R. A. (2000) Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation, *Journal of Clinical Investigation*, **106**, 551–559.

Lamm, M. E. (1997) Interaction of antigens and antibodies at mucosal surfaces, *Annual Reviews of Microbiology*, **51**, 311–340.

Lane, C., Knight, D., Burgess, S., Franklin, P., Horak, F., Legg, J., Moeller, A. & Stick, S. (2004) Epithelial inducible nitric oxide synthase activity is the major determinant of nitric oxide concentration in exhaled breath, *Thorax*, **59**, 757–760.

Laxmanan, S., Robertson, S. W., Wang, E., Lau, J. S., Briscoe, D. M. & Mukhopadhyay, D. (2005) Vascular endothelial growth factor impairs the functional ability of dendritic cells through Id pathways, *Biochemical and Biophysical Research Communications*, **334**, 193–198.

Lindh, E. (1975) Increased risistance of immunoglobulin A dimers to proteolytic degradation after binding of secretory component, *Journal of Immunology*, **114**, 284–286.

Martin, J. G., Suzuki, M., Maghni, K., Pantano, R., Ramos-Barbon, D., Ihaku, D., Nantel, F., Denis, D., Hamid, Q. & Powell, W. S. (2002) The immunomodulatory actions of prostaglandin E2 on allergic airway responses in the rat, *Journal of Immunology*, **169**, 3963–3969.

Matsukawa, A., Lukacs, N. W., Standiford, T. J., Chensue, S. W. & Kunkel, S. L. (2000) Adenoviral-mediated overexpression of monocyte chemoattractant protein-1 differentially alters the development of Th1 and Th2 type responses in vivo, *Journal of Immunology*, **164**, 1699–1704.

Mayer, L. & Shlien, R. (1987) Evidence for function of Ia molecules on gut epithelial cells in man, *Journal of Experimental Medicine*, **166**, 1471–1483.

McWilliam, A. S., Napoli, S., Marsh, A. M., Pemper, F. L., Nelson, D. J., Pimm, C. L., Stumbles, P. A., Wells, T. N. & Holt, P. G. (1996) Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli, *Journal of Experimental Medicine*, **184**, 2429–2432.

Mellor, A. L., Keskin, D. B., Johnson, T., Chandler, P. & Munn, D. H. (2002) Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses, *Journal of Immunology*, **168**, 3771–3776.

Monteiro, R. C. & Van De Winkel, J. G. (2003) IgA Fc receptors, *Annual Review of Immunology*, **21**, 177–204.

Moon, B. G., Takaki, S., Miyake, K. & Takatsu, K. (2004) The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production, *Journal of Immunology*, **172**, 6020–6029.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. (2001) Interleukin-10 and the interleukin-10 receptor, *Annual Review of Immunology*, **19**, 683–765.

Mori, A., Suko, M., Kaminuma, O., Inoue, S., Ohmura, T., Nishizaki, Y., Nagahori, T., Asakura, Y., Hoshino, A., Okumura, Y., Sato, G., Ito, K. & Okudaira, H. (1996) IL-15 promotes cytokine production of human T helper cells, *Journal of Immunology*, **156**, 2400–2405.

Mueller, D. L., Jenkins, M. K. & Schwartz, R. H. (1989) Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy, *Annual Reviews of Immunology*, **7**, 445–480.

Muller, F., Aukrust, P., Nilssen, D. E. & Froland, S. S. (1995) Reduced serum level of transforming growth factor-beta in patients with IgA deficiency, *Clinical Immunology and Immunopathology*, **76**, 203–208.

Muro, S., Taha, R., Tsicopoulos, A., Olivenstein, R., Tonnel, A. B., Christodoulopoulos, P., Wallaert, B. & Hamid, Q. (2001) Expression of IL-15 in inflammatory pulmonary diseases, *Journal of Allergy and Clinical Immunology*, **108**, 970–975.

Neutra, M. R., Frey, A. & Kraehenbuhl, J. P. (1996) Epithelial M cells: gateways for mucosal infection and immunization, *Cell*, **86**, 345–348.

Niess, J. H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B. A., Vyas, J. M., Boes, M., Ploegh, H. L., Fox, J. G., Littman, D. R. & Reinecker, H. C. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance, *Science*, **307**, 254–258.

Oei, E., Kalb, T., Beuria, P., Allez, M., Nakazawa, A., Azuma, M., Timony, M., Stuart, Z., Chen, H. & Sperber, K. (2004) Accessory cell function of airway epithelial cells, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, **287**, L318–L331.

Oppenheimer-Marks, N., Kavanaugh, A. F. & Lipsky, P. E. (1994) Inhibition of the transendothelial migration of human T lymphocytes by prostaglandin E2, *Journal of Immunology*, **152**, 5703–5713.

Otsuki, Y., Ito, Y. & Magari, S. (1989) Lymphocyte subpopulations in high endothelial venules and lymphatic capillaries of bronchus-associated lymphoid tissue (BALT) in the rat, *American Journal of Anatomy*, **184**, 139–146.

Paine, R., 3rd, Chavis, A., Gaposchkin, D., Christensen, P., Mody, C. H., Turka, L. A. & Toews, G. B. (1992) A factor secreted by a human pulmonary alveolar epithelial-like cell line blocks T-cell

proliferation between G1 and S phase, *American Journal of Respiratory Cell and Molecular Biology*, **6**, 658–666.

Paolucci, C., Rovere, P., De Nadai, C., Manfredi, A. A. & Clementi, E. (2000) Nitric oxide inhibits the tumor necrosis factor alpha -regulated endocytosis of human dendritic cells in a cyclic GMP-dependent way, *Journal of Biological Chemistry*, **275**, 19638–19644.

Papi, A., Stanciu, L. A., Papadopoulos, N. G., Teran, L. M., Holgate, S. T. & Johnston, S. L. (2000) Rhinovirus infection induces major histocompatibility complex class I and costimulatory molecule upregulation on respiratory epithelial cells, *Journal of Infectious Diseases*, **181**, 1780–1784.

Pettit, A. R., MacDonald, K. P., O'Sullivan, B. & Thomas, R. (2000) Differentiated dendritic cells expressing nuclear RelB are predominantly located in rheumatoid synovial tissue perivascular mononuclear cell aggregates, *Arthritis and Rheumatism*, **43**, 791–800.

Pichavant, M., Charbonnier, A. S., Taront, S., Brichet, A., Wallaert, B., Pestel, J., Tonnel, A. B. & Gosset, P. (2005) Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment, *Journal of Allergy and Clinical Immunology*, **115**, 771–778.

Pichavant, M., Taront, S., Jeannin, P., Breuilh, L., Charbonnier, A. S., Spriet, C., Fourneau, C., Corvaia, N., Heliot, L., Brichet, A., Tonnel, A. B., Delneste, Y. & Gosset, P. (2006) Impact of bronchial epithelium on dendritic cell migration and function: modulation by the bacterial motif KpOmpA, *Journal of Immunology*, **177**, 5912–5919.

Pilette, C., Ouadrhiri, Y., Godding, V., Vaerman, J. P. & Sibille, Y. (2001) Lung mucosal immunity: immunoglobulin-A revisited, *European Respiratory Journal*, **18**, 571–588.

Ranges, G. E., Figari, I. S., Espevik, T. & Palladino, M. A., Jr. (1987) Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha, *Journal of Experimental Medicine*, **166**, 991–998.

Regnier, M., Patwardhan, A., Scheynius, A. & Schmidt, R. (1998) Reconstructed human epidermis composed of keratinocytes, melanocytes and Langerhans cells, *Medical and Biological Engineering and Computing*, **36**, 821–824.

Reibman, J., Hsu, Y., Chen, L. C., Bleck, B. & Gordon, T. (2003) Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter, *American Journal of Respiratory Cell and Molecular Biology*, **28**, 648–654.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J. P. & Ricciardi-Castagnoli, P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria, *Nature Immunology*, **2**, 361–367.

Riedl, E., Stockl, J., Majdic, O., Scheinecker, C., Knapp, W. & Strobl, H. (2000) Ligation of E-cadherin on in vitro-generated immature Langerhans-type dendritic cells inhibits their maturation, *Blood*, **96**, 4276–4284.

Rimmelzwaan, G. F., Boon, A. C., Geelhoed-Mieras, M. M., Voeten, J. T., Fouchier, R. A. & Osterhaus, A. D. (2004) Human airway epithelial cells present antigen to influenza virus-specific CD8+ CTL inefficiently after incubation with viral protein together with ISCOMATRIX, *Vaccine*, **22**, 2769–2775.

Rincon, M., Anguita, J., Nakamura, T., Fikrig, E. & Flavell, R. A. (1997) Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells, *Journal of Experimental Medicine*, **185**, 461–469.

Roghanian, A., Williams, S. E., Sheldrake, T. A., Brown, T. I., Oberheim, K., Xing, Z., Howie, S. E. & Sallenave, J. M. (2006) The antimicrobial/elastase inhibitor elafin regulates lung dendritic cells and adaptive immunity, *American Journal of Respiratory Cell and Molecular Biology*, **34**, 634–642.

Rossi, G. A., Sacco, O., Balbi, B., Oddera, S., Mattioni, T., Corte, G., Ravazzoni, C. & Allegra, L. (1990) Human ciliated bronchial epithelial cells: expression of the HLA-DR antigens and of the HLA-DR alpha gene, modulation of the HLA-DR antigens by gamma-interferon and antigen-presenting function in the mixed leukocyte reaction, *American Journal of Respiratory Cell and Molecular Biology*, **3**, 431–439.

Sacco, O., Lantero, S., Scarso, L., Galietta, L. J., Spallarossa, D., Silvestri, M. & Rossi, G. A. (1999) Modulation of HLA-DR antigen and ICAM-1 molecule expression on airway epithelial cells by sodium nedocromil, *Annals of Allergy, Asthma and Immunology*, **83**, 49–54.

Salik, E., Tyorkin, M., Mohan, S., George, I., Becker, K., Oei, E., Kalb, T. & Sperber, K. (1999) Antigen trafficking and accessory cell function in respiratory epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **21**, 365–379.

Salvi, S., Semper, A., Blomberg, A., Holloway, J., Jaffar, Z., Papi, A., Teran, L., Polosa, R., Kelly, F., Sandstrom, T., Holgate, S. & Frew, A. (1999) Interleukin-5 production by human airway epithelial cells, *American Journal of Respiratory Cell and Molecular Biology*, **20**, 984–991.

Sauty, A., Dziejman, M., Taha, R. A., Iarossi, A. S., Neote, K., Garcia-Zepeda, E. A., Hamid, Q. & Luster, A. D. (1999) The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells, *Journal of Immunology*, **162**, 3549–3558.

Seder, R. A. (1996) High-dose IL-2 and IL-15 enhance the in vitro priming of naive CD4+ T cells for IFN-gamma but have differential effects on priming for IL-4, *Journal of Immunology*, **156**, 2413–2422.

Sim, G. K., Rajaserkar, R., Dessing, M. & Augustin, A. (1994) Homing and in situ differentiation of resident pulmonary lymphocytes, *International Immunology*, **6**, 1287–1295.

Sminia, T., van der Brugge-Gamelkoorn, G. J. & Jeurissen, S. H. (1989) Structure and function of bronchus-associated lymphoid tissue (BALT), *Critical Reviews in Immunology*, **9**, 119–150.

Smits, H. H., de Jong, E. C., Schuitemaker, J. H., Geijtenbeek, T. B., van Kooyk, Y., Kapsenberg, M. L. & Wierenga, E. A. (2002) Intercellular adhesion molecule-1/LFA-1 ligation favors human Th1 development, *Journal of Immunology*, **168**, 1710–1716.

Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishii, T., Sugimoto, M., Araki, S., Tominaga, A., Yamaguchi, N. & Takatsu, K. (1989) Transforming growth factor beta induces IgA production and acts additively with interleukin 5 for IgA production, *Journal of Experimental Medicine*, **170**, 1415–1420.

Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gorman, D., Zurawski, S., Abrams, J., Menon, S., McClanahan, T., de Waal-Malefyt, R., Bazan, F., Kastelein, R. A. & Liu, Y. J. (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP, *Nature Immunology*, **3**, 673–680.

Stanciu, L. A., Bellettato, C. M., Laza-Stanca, V., Coyle, A. J., Papi, A. & Johnston, S. L. (2006) Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines, *Journal of Infectious Diseases*, **193**, 404–412.

Stick, S. M. & Holt, P. G. (2003) The airway epithelium as immune modulator: the LARC ascending, *American Journal of Respiratory Cell and Molecular Biology*, **28**, 641–644.

Stumbles, P. A., Thomas, J. A., Pimm, C. L., Lee, P. T., Venaille, T. J., Proksch, S. & Holt, P. G. (1998) Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity, *Journal of Experimental Medicine*, **188**, 2019–2031.

Subudhi, S. K., Alegre, M. L. & Fu, Y. X. (2005) The balance of immune responses: costimulation verse coinhibition, *Journal of Molecular Medicine*, **83**, 193–202.

Suda, T., Sato, A., Sugiura, W. & Chida, K. (1995) Induction of MHC class II antigens on rat bronchial epithelial cells by interferon-gamma and its effect on antigen presentation, *Lung*, **173**, 127–137.

Sue-Chu, M., Karjalainen, E. M., Altraja, A., Laitinen, A., Laitinen, L. A., Naess, A. B., Larsson, L. & Bjermer, L. (1998) Lymphoid aggregates in endobronchial biopsies from young elite cross-country skiers, *American Journal of Respiratory and Critical Care Medicine*, **158**, 597–601.

Suh, W. K., Gajewska, B. U., Okada, H., Gronski, M. A., Bertram, E. M., Dawicki, W., Duncan, G. S., Bukczynski, J., Plyte, S., Elia, A., Wakeham, A., Itie, A., Chung, S., Da Costa, J., Arya, S., Horan, T.,

Campbell, P., Gaida, K., Ohashi, P. S., Watts, T. H., Yoshinaga, S. K., Bray, M. R., Jordana, M. & Mak, T. W. (2003) The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses, *Nature Immunology*, **4**, 899–906.

Taga, K. & Tosato, G. (1992) IL-10 inhibits human T cell proliferation and IL-2 production, *Journal of Immunology*, **148**, 1143–1148.

Tango, M., Suzuki, E., Gejyo, F. & Ushiki, T. (2000) The presence of specialized epithelial cells on the bronchus-associated lymphoid tissue (BALT) in the mouse, *Archives of Histology and Cytology*, **63**, 81–89.

Tazi, A., Bouchonnet, F., Grandsaigne, M., Boumsell, L., Hance, A. J. & Soler, P. (1993) Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers, *Journal of Clinical Investigation*, **91**, 566–576.

Teitelbaum, R., Schubert, W., Gunther, L., Kress, Y., Macaluso, F., Pollard, J. W., McMurray, D. N. & Bloom, B. R. (1999) The M cell as a portal of entry to the lung for the bacterial pathogen *Mycobacterium tuberculosis, Immunity*, **10**, 641–650.

Tschernig, T. & Pabst, R. (2000) Bronchus-associated lymphoid tissue (BALT) is not present in the normal adult lung but in different diseases, *Pathobiology*, **68**, 1–8.

Upham, J. W., Denburg, J. A. & O'Byrne, P. M. (2002) Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects, *Clinical and Exprimental Allergy*, **32**, 818–823.

van Ginkel, F. W., Wahl, S. M., Kearney, J. F., Kweon, M. N., Fujihashi, K., Burrows, P. D., Kiyono, H. & McGhee, J. R. (1999) Partial IgA-deficiency with increased Th2-type cytokines in TGF-beta 1 knockout mice, *Journal of Immunology*, **163**, 1951–1957.

van Rijt, L. S., Jung, S., Kleinjan, A., Vos, N., Willart, M., Duez, C., Hoogsteden, H. C. & Lambrecht, B. N. (2005) In vivo depletion of lung $CD11c^+$ dendritic cells during allergen challenge abrogates the characteristic features of asthma, *Journal of Experimental Medicine*, **201**, 981–991.

van Vlasselaer, P., Punnonen, J. & de Vries, J. E. (1992) Transforming growth factor-beta directs IgA switching in human B cells, *Journal of Immunology*, **148**, 2062–2067.

Vignola, A. M., Chiappara, G., Siena, L., Bruno, A., Gagliardo, R., Merendino, A. M., Polla, B. S., Arrigo, A. P., Bonsignore, G., Bousquet, J. & Chanez, P. (2001) Proliferation and activation of bronchial epithelial cells in corticosteroid-dependent asthma, *Journal of Allergy and Clinical Immunology*, **108**, 738–746.

Wang, A., Yokosaki, Y., Ferrando, R., Balmes, J. & Sheppard, D. (1996) Differential regulation of airway epithelial integrins by growth factors, *American Journal of Respiratory Cell and Molecular Biology*, **15**, 664–672.

Wang, Y. H., Ito, T., Wang, Y. H., Homey, B., Watanabe, N., Martin, R., Barnes, C. J., McIntyre, B. W., Gilliet, M., Kumar, R., Yao, Z. & Liu, Y. J. (2006) Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells, *Immunity*, **24**, 827–838.

Watanabe, N., Hanabuchi, S., Soumelis, V., Yuan, W., Ho, S., de Waal Malefyt, R. & Liu, Y. J. (2004) Human thymic stromal lymphopoietin promotes dendritic cell-mediated $CD4^+$ T cell homeostatic expansion, *Nature Immunology*, **5**, 426–434.

Yang, D., Chertov, O., Bykovskaia, S. N., Chen, Q., Buffo, M. J., Shogan, J., Anderson, M., Schroder, J. M., Wang, J. M., Howard, O. M. & Oppenheim, J. J. (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6, *Science*, **286**, 525–528.

Ying, S., O'Connor, B., Ratoff, J., Meng, Q., Mallett, K., Cousins, D., Robinson, D., Zhang, G., Zhao, J., Lee, T. H. & Corrigan, C. (2005) Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity, *Journal of Immunology*, **174**, 8183–8190.

Zan, H., Cerutti, A., Dramitinos, P., Schaffer, A. & Casali, P. (1998) CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu → S alpha and sequential S mu → S gamma, S gamma → S alpha DNA recombination, *Journal of Immunology*, **161**, 5217–5225.

Zegarra-Moran, O., Folli, C., Manzari, B., Ravazzolo, R., Varesio, L. & Galietta, L. J. (2004) Double mechanism for apical tryptophan depletion in polarized human bronchial epithelium, *Journal of Immunology*, **173**, 542–549.

12

Interactions of Respiratory Viruses with the Epithelium

Umadevi S. Sajjan, Dawn C. Newcomb, Toby C. Lewis, Christine S. Stahle and **Marc B. Hershenson**
University of Michigan, Ann Arbor, Michigan, USA

12.1 Introduction

Respiratory viral infections of the airway epithelium are responsible for significant morbidity and mortality, especially in children. Emerging infections, such as severe acute respiratory syndrome coronavirus and avian influenza, could have a significant impact on human health. Yet, the influence of respiratory viral infections may be most strongly felt in individuals with pre-existing airway diseases, particularly asthma and chronic obstructive pulmonary disease (COPD). Newer, more sensitive techniques for the diagnosis of respiratory viral infection, namely polymerase chain reaction (PCR), have pinpointed respiratory viruses, particularly rhinoviruses, as the main cause of asthma exacerbations, and viral infection has been associated with approximately half of all COPD exacerbations.

This chapter will describe the viruses responsible for clinically-important infections of the respiratory tract, as well as those responsible for exacerbations of asthma and COPD. Elements of the innate immune response of the lung will be reviewed, including the release of antimicrobial substances and the induction of pro-inflammatory cytokines and chemokines. Next, we will focus on the response of the airway epithelium to rhinovirus (RV), respiratory syncytial virus (RSV) and influenza. Evidence for the potential role of these viruses in the development of asthma will be considered. Finally, we will discuss potential cooperative effects between viral infection and allergic sensitization, bacterial infection and air pollution.

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

12.2 Causes of bronchiolitis and community-acquired pneumonia in infants, children and older adults

Bronchiolitis is the most common lower respiratory tract syndrome affecting young children. In developed countries, the case fatality rate among previously healthy children is low, about 2 per 100 000 in the USA (Holman *et al*., 2003). However, in the USA, as many as 1 per cent of all infants are hospitalized for bronchiolitis (Kim *et al*., 1973). More than 700 000 infants visit USA emergency departments each year because of bronchiolitis, and approximately one-third of these are admitted to the hospital (Leader and Kohlhase, 2003)

RSV is the most common underlying viral infection and has been isolated from 50 to 75 per cent of children younger than two years of age hospitalized with bronchiolitis (Glezen *et al*., 1986). Other common respiratory viral pathogens, such as influenza, parainfluenza, and adenovirus, have been isolated from children with bronchiolitis (Loda *et al*., 1968). Recent investigations have shown that some infants with bronchiolitis may be infected with RV (Korppi *et al*., 2004) or human metapneumovirus (Freymouth *et al*., 2003).

The causative agent of community-acquired pneumonia in children differs according to the age of the patient. In neonates and infants up to three months of age, bacteria are the most common pathogens, with Group B beta-haemolytic *Streptococcus* dominating in the newborn period and *Streptococcus pneumoniae* predominant thereafter. Parainfluenza virus 3 and RSV are also included in this age group (McIntosh, 2002). Viruses are the predominant pathogens in the 4 months to 4 years group, with RSV being the most common cause. Parainfluenza viruses, influenza virus, adenovirus, and RV have also been isolated from children with pneumonia of this age group (McIntosh, 2002).

Viral pneumonia is relatively uncommon during the re-infections of young adulthood. However, with advancing age and the inevitable development of co-morbidities, viruses once again cause serious illness and pneumonia. Although acute respiratory infection rates steadily decrease with advancing age, rates of hospitalization and death increase substantially in persons aged greater than 60 years. Multiple factors, such as declines in respiratory and immune function, likely contribute. Immune dysfunction and may impair viral clearance, allowing spread of the virus to lower airway, with increased inflammation.

Studies of community-acquired pneumonia in adults indicate a viral aetiology in up to one-quarter of cases, with influenza virus being the most common virus (File, 2003). RSV is the second-most common cause of viral pneumonia in older persons. Finally, metapneumovirus, parainfluenza, coronavirus and RV have been identified (Falsey and Walsh, 2006).

12.3 Viruses implicated in the exacerbation of asthma and COPD

Recent studies examining the association between viral infection of the upper respiratory tract and exacerbations of chronic airway disease have benefited from the advent of PCR, which is significantly more sensitive than viral culture in the diagnosis of infection. Epidemiologic studies have uncovered a strong association between viral infections, especially those caused by RV, and exacerbations of asthma and COPD. Viral infections trigger nearly 80 per cent of asthma exacerbations in children (Johnston *et al*., 1995) and adults (Nicholson *et al*., 1993). In exacerbations of asthma in children aged 9–11 years over a 13-month period,

viruses were detected in 77 per cent of episodes. Picornaviruses (i.e., RV) were detected in 65 per cent of these viral infections, coronaviruses in 17 per cent, influenza and parainfluenza viruses each in 9 per cent and RSV in 5 per cent (Johnston *et al*., 1995). As expected, the involvement of specific viruses may vary with the season, with a recent study detecting RV in 82 per cent of all children admitted to an emergency room for acute asthma between the months of January and July (Kling *et al*., 2005). In adults, colds were reported in 80 per cent of all asthma exacerbations, and 46 per cent of these episodes were accompanied by confirmed viral infection, the majority of which were ascribed to RV (61 per cent), followed in frequency by coronaviruses, parainfluenza virus, influenza virus and RSV (Nicholson *et al*., 1993).

Exacerbations of COPD have long been associated with bacterial infection, most commonly intracellular non-typeable *Hemophilus influenzae* (NTHI). Exacerbation has also been associated with the acquisition of new strains of *H. influenzae, Moraxella catarrhalis* and *Streptococcus pneumoniae* (Sethi *et al*., 2002) and, in patients with severely reduced lung function, *Enterobacter* and *Pseudomonas* species (Eller *et al*., 1998). On the other hand, between 27 to 56 per cent of exacerbations are associated with respiratory viral infections, and RV is the most common virus isolated (Greenberg *et al*., 2000; Seemungal *et al*., 2001; Rohde *et al*., 2003). In a cohort of subjects of whom greater than 90 per cent had been vaccinated against influenza, coronaviruses (29 per cent), parainfluenza viruses and picornaviruses (23 per cent each) were most commonly identified (Greenberg *et al*., 2000). In two studies, RV predominated (Seemungal *et al*., 2001; Rohde *et al*., 2003). By virtue of their relative prevalence and importance in disease pathogenesis, the following discussion of interactions between respiratory viruses and the epithelium will focus on RV, RSV and influenza virus.

12.4 Upper and lower respiratory tract infections

As noted above, many exacerbations of asthma and COPD are associated with infection by viruses typically associated with upper, rather than lower, respiratory tract infection. This point not only applies to RV and coronaviruses, causes of the common cold, but also other viruses as well. For example, RSV induces only mild-to-moderate upper respiratory tract symptoms in older children and adults. How, then, do viruses typically associated with upper respiratory tract infections induce exacerbations of lower respiratory tract disease?

Recent studies examining the pathogenesis of rhinovirus infections are instructive. Because RV replication is optimal at 33–35 °C, infections were thought to be restricted to upper airway tissues. Until recently, RV had not been cultured from lower airway secretions. Accordingly, different theories were proposed to explain how signals from the upper respiratory tract might alter lower airway function. These included the release of pro-inflammatory cytokines from the nasal epithelium into the systemic circulation, and exposure of nasal epithelial sensory parasympathetic fibres, leading to bronchoconstriction via increased efferent vagal activity.

It has recently become clear, however, that infections of the upper respiratory tract may be accompanied by the entry of virus into lower respiratory tract epithelial cells. RV can replicate in lower airway cells even at core temperature, though greater viral yields are obtained at cooler temperatures (Schroth *et al*., 1999). Also, temperatures of the large airway are 33–35 °C during resting breathing at room temperature (McFadden *et al*., 1985). Thus,

conditions in the lower airway may be favourable for RV replication. After experimental infection, RV RNA has been detected in lower airway secretions (Gern *et al*., 1997). Four days after experimental RV infection, immunohistochemistry of the segmental bronchial epithelium demonstrates the presence of RV16 capsid protein, though some samples showed only one or two cells positive for virus (Mosser *et al*., 2005). RV infection increases lower airway intercellular adhesion molecule (ICAM)-1 expression (Grunberg *et al*., 2000). Recently, a case of an immunosuppressed lung transplant recipient with chronic RV infection of the lower airway was described (Kaiser *et al*., 2006). Taken together, these findings suggest that RV grows in the lower airway, although the quantity of viral replication in the lower airway may be limited. Accordingly, in our subsequent discussions of pathogenesis, we will assume that respiratory viruses infect both upper and lower respiratory tract epithelial cells.

12.5 The innate immune response

The airway epithelium functions as the major interface between the host and external environment. Rather than serve a passive role, the epithelium releases antimicrobial substances as well as pro-inflammatory cytokines and chemokines, leading to clearance of micro-organisms and activation of the adaptive immune system.

The first phase of the response is recognition of micro-organisms by their pathogen-associated molecular patterns (PAMPs). PAMPs, in turn, are recognized by pattern-recognition receptors which consist primarily of Toll-like receptors (TLRs) on the cell surface. Ten mammalian TLRs have been described, each with specific ligands (Chapter 9). Different TLRs use different combinations of adaptor proteins to generate intracellular signals. Myeloid differentiation (MyD)-88 appears to function as an adaptor protein for all TLRs except TLR3, which employs TRIF (Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β) (also called TICAM-1, for Toll/interleukin-1 receptor adaptor molecule-1). TLR3 mediates immune responses to double-stranded (ds) RNA. TLR3 is expressed in airway epithelial cells (Sha *et al*., 2004), though the subcellular localization in differentiated polarized cells has not been resolved. TLR3 was recently identified in dendritic cell endocytic multivesicular bodies (Matsumoto *et al*., 2003). During replication, single-stranded (ss) RNA viruses make ds-RNA intermediates, consistent with the notion that TLR3 mediates responses to RV, RSV, influenza virus, and other respiratory viruses. Other possible receptors for viral RNA include TLRs 7/8, which recognize ssRNA and signal through MyD88, cytoplasmic dsRNA-dependent protein kinase R, and the cytoplasmic RNA helicases retinoic-acid-inducible protein (RIG)-I and melanoma-differentiation-associated gene 5 (MDA5). Finally, TLR4, which is well-known for its recognition of lipopolysaccharide, also binds to the RSV glycoprotein F (Kurt-Jones *et al*., 2000; Monick *et al*., 2003).

Engagement of TLRs activates downstream signalling pathways leading to activation of nuclear factor (NF)-κB and interferon (IFN) regulatory factors (IRFs)-3 and -7, key transcription factors involved in the expression of cytokines, chemokines and IFNs. The TLR3 adaptor molecule TRIF/TICAM-1 consists of an N-terminal proline-rich domain, a Toll/IL-1 receptor (TIR) domain and C-terminal proline-rich domain. The N-terminal region of TRIF directly associates with tumour necrosis factor (TNF) receptor-associated factor (TRAF)-6, a ubiquitin ligase, and TANK-binding kinase (TBK)-1. (TANK stands for TRAF family member-associated NF-κB activator.) Following viral infection, the

association with TRAF6 leads to activation of the canonical IκB kinase (IKK) complex and NF-κB, which upregulates the transcription of pro-inflammatory genes such as IL-6, IL-1 and TNF-α. The recruitment of TBK-1 to the C-terminal region of TRIF initiates a signalling cascade that culminates in IRF-3 activation and the induction of IFN, RANTES and IP-10. Finally, other kinases required for the expression of cytokines, chemokines and IFNs may be activated nonspecifically upon binding and endocytosis of virus (see section 12.6 below).

Recognition of microorganisms by the airway epithelium leads to the release of antimicrobial substances as well as pro-inflammatory cytokines and chemokines. These antimicrobial substances include the collectins, defensins and nitric oxide. Collectins are collagen-binding lectins of the C-type lectin superfamily. The collectins involved in lung innate defence are surfactant protein (SP)-A, SP-D and mannose binding lectin (MBL). Each shares a carbohydrate recognition domain that is essential for binding to monosaccharide arrays present on the microbial surface. SP-A and SP-D bind and agglutinate micro-organisms and other particulate material entering the lungs, thereby promoting attachment, uptake, and killing of respiratory pathogens by alveolar macrophages. Like SP-A and SP-D, MBL can act directly as an opsonin by binding to carbohydrates on pathogens and then interacting with MBL receptors on phagocytic cells. By virtue of its structural resemblance to C1q, MBL can also trigger the opsonic activity of complement, resulting in deposition of C3b/inactivated C3b on targets and stimulation of phagocytic uptake via the C3 receptors CR1, CR3 and CR4 (Hickling *et al*., 2004).

Pulmonary infiltration after RSV infection is more severe in SP-A knockout mice, and co-administration of RSV with exogenous SP-A reduces viral titres and inflammatory cells in the lungs of SP-A knockout mice (LeVine *et al*., 1999). SP-A binds both RSV G and F glycoproteins (Ghildyal *et al*., 1999). SP-D binds G protein and inhibits RSV infection in vitro and in vivo (Hickling *et al*., 1999). Susceptibility to RSV infection in infants has been linked to polymorphisms in both the SP-A and SP-D genes (Lahti *et al*., 2002; Lofgren *et al*., 2002).

SP-A, SP-D and MBL bind and neutralize influenza A virus in vitro (Reading *et al*., 1997; Malhotra *et al*., 1994). Influenza strains with more haemagglutinin glycosylation sites are more sensitive to neutralization by SP-D and MBL and replicate poorly in mouse lungs compared to strains with fewer glycosylation sites (Reading *et al*., 1997). Growth of influenza A in the lungs is enhanced when saccharide inhibitors of collectins are included in the virus inoculum. Influenza A infection increases levels of SP-D and MBL in the bronchoalveolar lavage fluid of infected mice. Together, these results implicate SP-D, MBL and possibly SP-A as important components of the innate defence of the respiratory tract against influenza virus.

Defensins are small cationic microbicidal substances secreted into the epithelial lining fluid which serve to kill potential pathogens and neutralize inflammatory substances such as lipopolysaccharide. Based on their biochemical structure, defensins are divided into the α- and β-defensin subfamilies (Chapter 10). Human α-defensins 1–4 are major components of the human neutrophil azurophilic granules. Human β-defensins (HBDs)-1, 2, 3 and 4 are commonly found in epithelial cells (Schutte and McCray, 2002). RV infection of primary human airway epithelial cells induces HBD-2 mRNA and protein expression, and in vivo infection of normal human subjects with RV16 induces expression of HBD-2 mRNA and protein in nasal epithelial scrapings which correlates with viral titre (Proud *et al*., 2004). These data are consistent with the notion that HBD-2 plays a role in host defence to

RV infection. In addition to this antimicrobial function, HBD is selectively chemotactic for cells stably transfected to express human C-C chemokine receptor (CCR)-6, a chemokine receptor preferentially expressed by immature dendritic cells and memory T-cells (Yang *et al*., 1999). Finally, murine β-defensin 2 has been shown to function as an endogenous ligand for TLR-4 in immature dendritic cells (Biragyn *et al*., 2002).

Nitric oxide (NO) is produced by airway epithelial cells and critically involved in nonspecific (innate) and immunological host defence. NO, a gaseous nitrogen-centred inorganic radical, has antimicrobial actions against various pathogens via its cytotoxic or cytostatic effects. The relative deficiency of epithelial nitric oxide synthase (NOS)-2 in patients with cystic fibrosis appears to be a factor in the increased airway infections observed in these patients (Zheng *et al*., 2003). Although the importance of NO has been documented for host defence reactions against bacteria and fungi, its role in the pathogenesis of virus infections is only partly understood.

NO is synthesized by oxidative conversion of the amino acid L-arginine by NOS. Three NOS isoforms have been identified, and all three are expressed within the respiratory tract. NOS1 (nNOS) and NOS3 (eNOS) are primarily expressed in neuronal and endothelial cell types, respectively, and are highly dependent on increases in intracellular Ca^{2+} for enzyme activation. In the airway, nonadrenergic, noncholinergic nerve fibres express NOS1, and the NO generated is a major mediator of neural smooth muscle relaxation (Belvisi *et al*., 1992). In the bronchial epithelium, NOS3 is localized at the basal membrane of ciliary microtubules and mediates regulation of ciliary beat frequency (Li *et al*., 2000). NOS2 is expressed in human airway epithelial cells and is the primary source of NO in asthmatic airway (Guo *et al*., 2000). Expression of NOS2 is transcriptionally regulated in response to pro-inflammatory cytokines including IFNs. Other forms of regulation, including epigenetic, translational, post-translational and proteolytic, exist.

Antiviral effects of NO are known for many types of virus. With regard to respiratory viruses, RV infection induces expression of NOS2 in human respiratory epithelial cells in vitro and in vivo (Sanders *et al*., 2001), and NO reduces RV-induced cytokine production and viral replication in a human respiratory epithelial cell line (Sanders *et al*., 1998). Replication of RSV is inhibited in HEp-2 cells constitutively expressing NOS (Ali-Ahmad *et al*., 2003). NO inhibits SARS-coronavirus infection in vitro (Keyaerts *et al*., 2004). Finally, cultured CF airway epithelial cells are more susceptible to human parainfluenza virus-3 infection than normal cells, and overexpression of NOS2 or an NO donor protects the cells from virus (Zheng *et al*., 2003).

On the other hand, NO has little antiviral activity against some viruses, and may actually impair antiviral responses by suppressing Th1 functions. Further, NO-induced cytotoxicity via oxidative injury may cause cellular and organ dysfunction. Markedly improved outcome is observed for murine influenza A viral pneumonia following treatment with a NOS inhibitor, whereas placebo-treated mice showed evidence of peroxynitrite-mediated lung damage (Akaike *et al*., 1996). NO impairs the anti-influenza virus response of the host by suppressing Th1-dependent IFN-γ induction and tipping the Th1–Th2 balance toward Th2 domination (Karupiah *et al*., 1998). Similarly, in genetically deficient NOS2 (−/−) mice, hosts survived with little histopathologic evidence of pneumonitis, whereas infection in NOS2 (+/+) mice resulted in consolidating pneumonitis and death. NOS2 (+/+) mice treated with a NOS inhibitor demonstrated improved survival without affecting viral growth (Karupiah *et al*., 1998). Thus, the role of NO in immunological host responses against viruses is complex.

12.6 Rhinoviruses

RVs, members of the *Picornaviridae* family, are the most common causes of upper respiratory tract infections ('colds') in humans and, accordingly, are the most common causes of asthma and COPD exacerbations (see above). The RV genome is composed of a single strand of positive-sense RNA (meaning it can be translated directly by the ribosome) enclosed in a small tetrahedral capsid. There are more than 100 serotypes of RV which can be classified into two major groups based on their cellular receptor. The major subgroup of RVs contains approximately 90 per cent of the serotypes (e.g., RV14, -16 and -39) and utilizes ICAM-1, also known as CD54, as the airway epithelial cell receptor. The remaining serotypes (e.g., RV1B and -2) use the family of low density lipoprotein receptors (LDL-R) as their means of entry.

The precise mechanisms by which RV induces asthma or COPD exacerbations are unknown. Typically, RV infects small clusters of cells in the epithelial layer (Mosser *et al*., 2005). While virus-induced cytotoxicity has been well documented for influenza, parainfluenza, adenovirus and RSV infections, RVs induce minimal, if any, cytotoxicity (Fraenkel *et al*., 1995). While a recent study has shown cytotoxicity in RV-infected subconfluent BEAS-2B cells (Bossios *et al*., 2005), epithelial cell shedding is unlikely to contribute to RV-induced exacerbations of chronic airway disease.

Instead, numerous studies suggest a role for C-X-C chemokines with the neutrophil-attractant Glu-Leu-Arg (ELR) motif in the pathogenesis of asthma and COPD exacerbations. ELR-positive C-X-C chemokines include interleukin (IL)-8 (also known as C-X-C ligand-8, or CXCL8), epithelial neutrophil attractant (ENA)-78 (CXCL5) and growth-related oncogene (GRO)-α (CXCL1). IL-8 and neutrophils are found in the nasal secretions and sputum of patients with RV-induced asthma exacerbations (Grunberg *et al*., 1997a, 1997b; Pizzichini *et al*., 1998). Further, the number of neutrophils correlates with the level of IL-8 (Pizzichini *et al*., 1998). Neutrophil number, IL-8 and ENA-78 are increased in the sputum and airway of patients with exacerbations of asthma (Norzila *et al*., 2000; Ordonez *et al*., 2000) and COPD (Bhowmik *et al*., 2000; Qiu *et al*., 2003). RV induces IL-8, ENA-78 and GRO-α expression in cultured airway epithelial cells (Subauste *et al*., 1995; Schroth *et al*., 1999; Griego *et al*., 2000; Newcomb *et al*., 2005). After RV16 infection, asthmatic patients show increased levels of IL-8 in their nasal lavage which correlates with the level of airway responsiveness (Grunberg *et al*., 1997b), in contrast to unaffected individuals in whom IL-8 does not increase (de Kluijver *et al*., 2003). Together, these data suggest that RV infection of airway epithelial cells may potentiate pre-existing inflammation by enhancing the production of neutrophil chemoattractants and neutrophilic airway inflammation.

It is also conceivable that RV infection induces lymphocytic and/or eosinophilic inflammation. It has recently been shown that γ -interferon inducible protein (IP)-10/CXCL10 is produced in response to RV infection in vitro and in vivo (Spurrell *et al*., 2005). IP-10 is a chemoattractant for activated type 1 T-lymphocytes and natural killer T cells which also suppresses eosinophil infiltration. In one study of experimental RV infection in asthmatic patients, airway T-cells increased while eosinophils decreased (Grunberg *et al*., 2001), consistent with a functional role for IP-10. On the other hand, evidence also exists for the role of eosinophils in the pathogenesis of viral-induced asthma exacerbations. Eosinophils and eosinophil cationic protein have been detected in the airway following experimental infection (Fraenkel *et al*., 1995; Grunberg *et al*., 1997a) and RV infection increases airway epithelial cell production of the eosinophil chemoattractant RANTES (for regulated upon

activation, normal T-cell expressed and secreted, also known as C-C ligand 5) (Schroth *et al*., 1999; Gern *et al*., 2003).

RV infection also upregulates expression of its receptors, ICAM-1 (Sethi *et al*., 1997; Papi and Johnston, 1999) and LDL-R (Suzuki *et al*., 2001a). Finally, RV infection induces the expression of antiviral cytokines such as interferon (IFN)-β and -λ (Wark *et al*., 2005; Contoli *et al*., 2006), as well as many IFN-inducible genes (Chen *et al*., 2006).

Biochemical signalling mechanisms involved in the innate immune response to RV infection have recently begun to be elucidated. Each step in the viral life cycle is capable of activating a discrete signalling pathway (Figure 12.1). RVs infect human epithelial cells

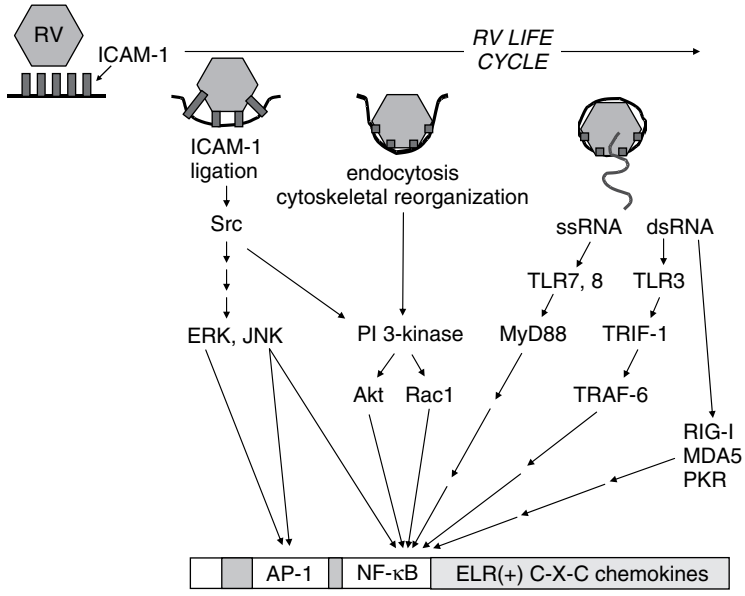


Figure 12.1 Steps in RV infection including ICAM engagement, endocytosis and RNA injection. Parallel signalling events are shown. dsRNA may activate the innate immune system via endocytic TLR3 receptors or cytoplasmic receptors including RIG-I, MDA5 or protein kinase R

via ceramide-enriched membrane platforms (Grassme *et al*., 2005). Bound RV is then localized to coated pits and internalized by clathrin-mediated endocytosis (Grunert *et al*., 1997; DeTulleo and Kirchhausen, 1998), a process which requires the GTPase dynamin 1 (DeTulleo and Kirchhausen, 1998). The mildly acidic pH of the endosome triggers uncoating and penetration. Using confocal microscopy, we have shown in airway epithelial cells that RV co-localizes with the tyrosine kinase Src, the p110β catalytic subunit of phosphatidylinositol (PI) 3-kinase and the serine-threonine kinase Akt in lipid rafts (Bentley *et al*., 2007). Activation of Src following ICAM-1 engagement induces phosphorylation of the PI 3-kinase p85 regulatory subunit, activation of PI 3-kinase, accumulation of 3-phosphorylated PI at the site of RV infection and Akt phosphorylation (Newcomb *et al*., 2005; Bentley *et al*., 2007), and PI 3-kinase activation is required and sufficient for subsequent NF-κB activation and chemokine expression (Newcomb *et al*., 2005). The Rho GTPase Rac1 is also activated during the cytoskeletal reorganization accompanying endocytosis and is required and

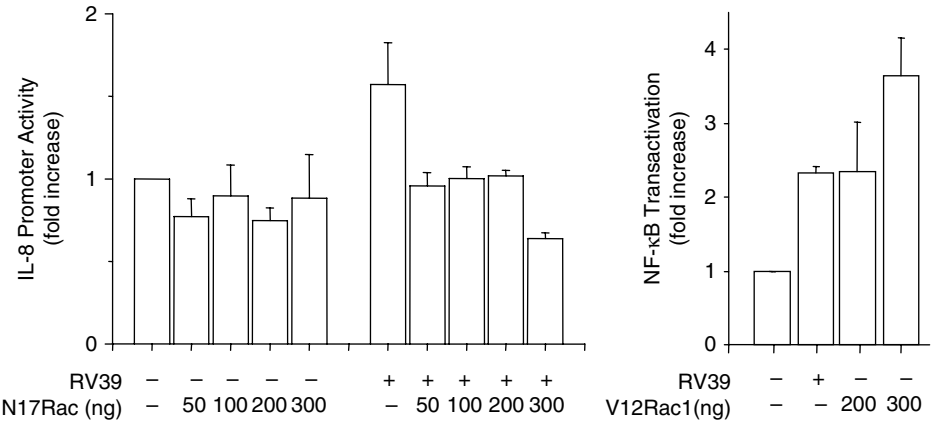


Figure 12.2 The Rho GTPase Rac1 is required for RV-induced IL-8 expression (left panel) and sufficient for NF-κB transactivation (right panel). 16HBE14o- human bronchial epithelial cells were transiently transfected with an IL-8 reporter plasmid (IL-8/Luc) and either a dominant-negative (N17Rac1) or constitutively-active (V12Rac1) Rac1

sufficient for IL-8 expression (Figure 12.2). In vascular endothelial and smooth muscle cells, ICAM-1 engagement activates signalling through extracellular signal-regulated kinase (ERK) (Lawson *et al*., 1999). ERK is activated minutes after RV infection of airway epithelial cells, and ERK activation is required for maximal RV-induced IL-8 expression (Newcomb *et al.,* 2007). Taken together, these data suggest that early events in the RV life cycle, i.e., binding and endocytosis, are sufficient to activate signalling pathways required for subsequent chemokine expression. Put another way, viral replication may not be necessary for at least a subset of RV-induced responses, perhaps explaining how RV can induce exacerbations of lower airway disease in the absence of abundant viral replication (Halperin *et al*., 1983). RV-induced activation of ERK enhances the response to tumor necrosis factor-α (Newcomb *et al.,* 2007), leading to additive or synergistic pro-inflammatory responses. An example of synergy between RV and a pro-asthmatic cytokine, IL-13, is shown in Figure 12.3.

Little is known about the transcription factors involved in the induction of chemokine and cytokine expression by RV in airway epithelial cells. RV infection activates NF-κB (Papi and Johnston, 1999; Newcomb *et al*., 2005). Expression of the rhinovirus 3C protease induces activator protein (AP)-1 transactivation (Funkhouser *et al*., 2004).

Ultraviolet (UV) irradiation inhibits viral replication and, therefore, can be used to determine the requirement of viral replication for inflammatory responses. UV irradiation inhibits, but does not abolish, RV39-induced early cytokine release in BEAS-2B cells (Griego *et al*., 2000). UV irradiation does not significantly reduce RV14-induced IL-8 expression in human tracheal epithelial cells (Suzuki *et al*., 2001b). RV39-induced IL-8 expression occurs independently of viral replication in MRC-5 fibroblasts (Kaul *et al*., 2000). Finally, bafilomycin, an inhibitor of vacuolar proton ATPases which promote the low endosomal pH needed for viral uncoating, decreases RV14-induced ICAM-1, but not IL-8, expression in human tracheal epithelial cells (Suzuki *et al*., 2001b). These studies are consistent with the notion that viral replication is not necessary for at least some RV-induced responses.

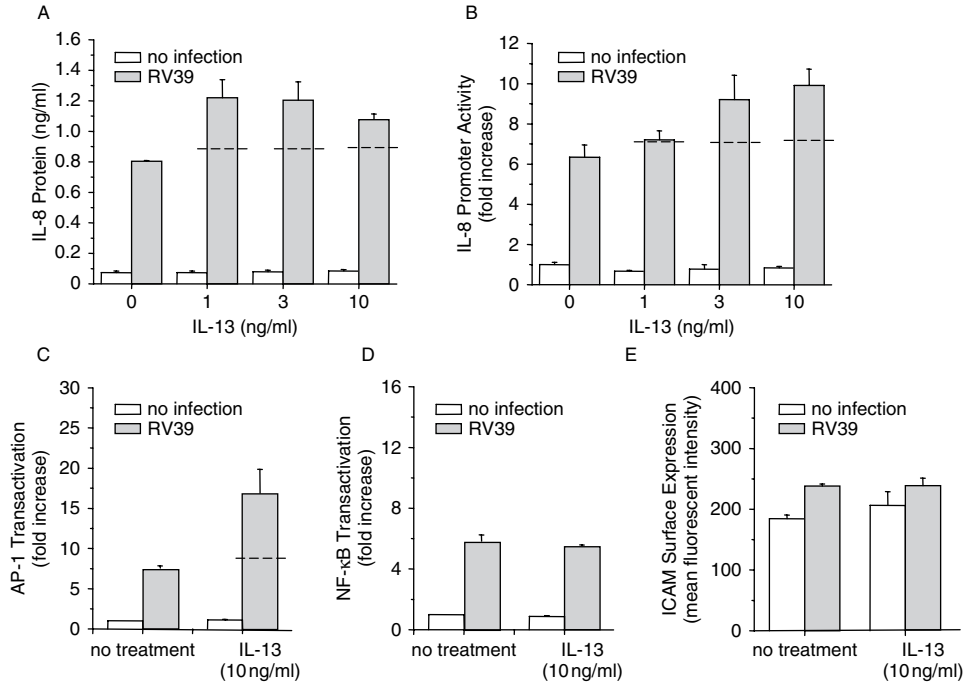


Figure 12.3 RV39 infection of 16HBE14o- human bronchial epithelial cells increases the level of IL-8 expression induced by IL-13, a pro-asthmatic cytokine. Cells were infected with RV39 and treated with IL-13. IL-13 alone had no effect on IL-8 protein expression, but combination with RV39 induced greater effects in IL-8 protein (panel A) and promoter activity (panel B). The synergistic increase in IL-8 was accompanied by a cooperative increase in AP-1 transactivation (panel C) but not NF-κB transactivation (panel D) or surface ICAM-1 expression (panel E)

Since viral replication, or even uncoating, may not be necessary for RV-induced IL-8 release, this implies that this aspect of the RV response may be nonspecific. In other words, RV-induced cytokine release may reflect the cellular response to ICAM-1 engagement and the endocytosis of receptor–ligand complexes, rather than a specific response to RV itself. If this is the case, the predominance of RV as a trigger for asthma and COPD exacerbations may simply reflect its prevalence compared to other respiratory viruses. Further, it is conceivable that additional stimuli, e.g., other viruses or even particulate matter, induce chemokine expression by similar mechanisms.

On the other hand, since the induction of IL-8 and other pro-inflammatory substances in response to RV infection appears to be biphasic in nature (Griego *et al.*, 2000), it is, perhaps, more likely that the early signalling events initiated by viral attachment and endocytosis are responsible for the early reaction to RV, to be followed by a second set of replication-dependent responses. Recent studies have examined the role of viral replication and viral RNA in RV-induced airway epithelial cell chemokine expression. Although RV is a single-stranded RNA virus, during replication it makes double-stranded (ds)-RNA intermediates. Toll-like receptor (TLR)-3, a type I transmembrane protein, mediates immune responses to dsRNA. Airway epithelial cells are activated by dsRNA (Gern *et al.*, 2003; Sha *et al.*,

2004), suggesting that TLR3 mediates RV-induced responses. We have recently found that, in primary mucociliary-differentiated airway epithelial cells, TLR3-specific siRNA inhibits RV-stimulated IL-8 production (Sajjan *et al*., 2006). Furthermore, HEK293 cells stably transfected with TLR3 produced high amounts of IL-8 in response to RV infection. These findings suggest that TLR3 is required for maximal RV-induced IL-8 expression. The roles of TLR7 and TLR8, which recognize single-stranded RNA, have not yet been tested. Similarly, the contribution of RIG-I and MDA5, cytoplasmic helicase proteins recently implicated in viral dsRNA recognition, have not been examined.

Another receptor for dsRNA is protein kinase R (PKR), which regulates NF-κB and other transcription factors. The PKR inhibitor 2-aminopurine blocks dsRNA-induced RANTES and IL-8 secretion (Gern *et al*., 2003), as well as RV-induced gene expression in primary human airway epithelial cells (Chen *et al*., 2006), suggesting that viral replication and PKR are required for maximal RV-induced responses. However, 2-aminopurine also inhibits ERK, Akt and other serine/threonine kinases. In our hands, both 2-aminopurine and a PKR mutant with a defective dsRNA binding motif-1 (Wu and Kaufman, 1996) attenuate but do not block RV39-induced IL-8 expression, suggesting the importance of other receptors for dsRNA (Figure 12.4).

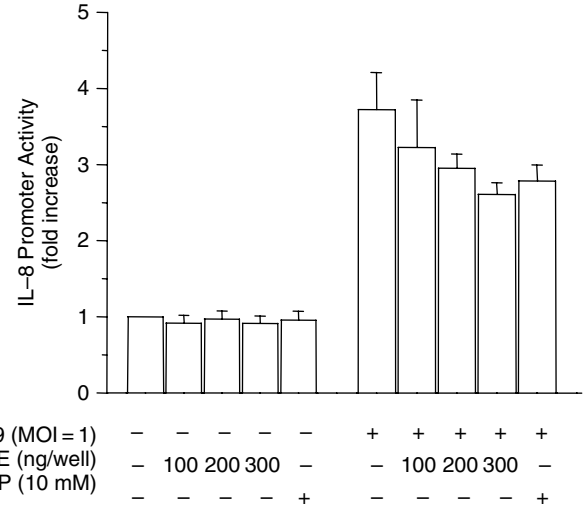


Figure 12.4 Inhibition of PKR does not abolish RV39-induced IL-8 promoter activity. To test for the requirement of dsRNA-dependent PKR for RV39-induced IL-8 expression, 16HBE14o- cells were co-transfected with IL-8/Luc and PKR-K64E, a PKR with a defective dsRNA binding motif (from R. Kaufman, University of Michigan). The mutant did not abolish RV39-induced IL-8 promoter activity. 2-aminopurine (2-AP) had a similar effect

Once released into the cytoplasm, translation of viral RNA is initiated. The picornavirus genome contains a 5′ and 3′ noncoding region and encodes a single polyprotein that is secondarily cleaved into mature proteins. The polyprotein is divided into three regions; P1, P2, and P3. P1 is cleaved into four mature capsid proteins, and the P2 and P3

regions comprise non-structural proteins that terminate host cell translation and promote viral replication. We have shown that a cleavage product of P3 called protease 3C is sufficient to increase IL-8 expression in human bronchial epithelial cells (Funkhouser *et al*., 2004).

Thus, there is a wealth of new information describing how RV induces airway epithelial cell chemokine expression. However, none of the above studies address the mechanism by which RV induces airway narrowing, the hallmark of asthma exacerbation. While RV has been noted to infect airway smooth muscle cells in vitro (Hakonarson *et al*., 1998), we are unaware of information demonstrating that RV infects airway smooth muscle in vivo. While the evidence that RV induces neutrophilic airway inflammation is compelling, at present little is known about the manner by which neutrophils contribute to airway narrowing in asthma or COPD. Activated neutrophils release a large array of inflammatory mediators (in particular tumour necrosis factor-α), oxygen radicals and proteases capable of inducing mucus production and airway remodelling.

Evidence has emerged that RVs are the most common cause of wheezing illness in the first year of life, and that RV-induced wheezing illness in the first year of life is the strongest predictor of subsequent third-year wheezing (Lemanske *et al*., 2005). It is therefore conceivable that RV may not only induce exacerbations of asthma, but also contribute to its primary pathogenesis. On the other hand, RV-associated wheezing may only denote an asthmatic predisposition. In support of this hypothesis, it has been shown that subjects who are low producers of IFN-γ in response to RV appear to be at greater risk for wheezing or having a severe respiratory infection. Peripheral blood monocytes from patients with asthma have a deficient type II IFN-γ response to RV (Papadopoulos *et al*., 2002), and the IFN-γ response correlates with FEV_1 and PD_{20} (the methacholine provocative dose for a 20 per cent decrease in FEV_1) (Brooks *et al*., 2003). Children with greater than or equal to two episodes of wheezing in infancy are less likely to have RV-induced IFN-γ responses at birth (Gern *et al*., 2006). Bronchial epithelial cells isolated from patients with asthma have been demonstrated to have an incomplete innate immune response to rhinovirus infection, with deficient type I interferon-β and type III interferon-λ production (Wark *et al*., 2005; Contoli *et al*., 2006). Thus, the host response, rather than the infecting organism, may be the best predictor of the future pattern of respiratory illness.

12.7 Respiratory syncytial virus

Human RSV is a negative sense, single-stranded RNA virus of the family *Paramyxoviridae*. As noted above, bronchiolitis is the most common lower respiratory tract syndrome affecting young children, with RSV being the most common underlying viral infection (Glezen *et al*., 1986). In developed countries, mortality is low, about 2 per 100 000 cases in the USA (Holman *et al*., 2003). However, RSV bronchiolitis is the primary cause of hospitalization in the first year of life in the USA (Kim *et al*., 1973). Premature babies born at 30–35 weeks of gestation and infants with cyanotic congenital heart disease are at particular risk.

The role of RSV in the pathogenesis of asthma has long been a source of debate. RSV infection is associated with increased bronchial reactivity and wheezing. Investigators have hypothesized that RSV infection skews the immune response towards an allergic phenotype. Data from the Tuscon Children's Respiratory Study showed that RSV lower respiratory tract infection in infants is associated with an increased risk of wheeze by age 6 years, but that the risk of wheezing decreases markedly thereafter and is not significant by age 13. There was

no association with atopic status (Stein *et al*., 1999). In contrast, another study examining the respiratory status of hospitalized infants with RSV showed increased wheezing and allergies compared to normal subjects at age 13 (Sigurs *et al*., 2005). The most likely explanation is that only a subset of infants, for example, those with pre-morbid differences in airway structure or function, or differences in the immune response, develop persistent wheeze in response to RSV infection. Studies have shown that children who wheeze during the first years of life have diminished lung function shortly after birth (Martinez *et al*., 1988). Children with measurable cord blood IFN responses to RSV are less likely to wheeze in their first year of life (Gern *et al*., 2006).

In contrast to RV, RSV infection results in the loss of cilia and sloughing of epithelial cells into the airway, leading to obstruction. The airway lumen is also packed with polymorphonuclear leukocytes, fibrin, lymphocytes and mucus. The airway wall is edematous and infiltrated with inflammatory cells. Interestingly, the immune response to RSV is responsible for a substantial share of the clinical illness. As evidence of this, mice depleted of CD4 and CD8 T cells have no discernible illness upon RSV infection, despite persistent viral infection for several weeks (Graham *et al*., 1991). Further, primary human mucociliary-differentiated airway epithelial cells cultured in the absence of immune cells show little cytotoxicity after RSV infection (Zhang *et al*., 2002).

The robust innate immune response to RSV infection has three components: surfactant proteins, chemokines and Toll-like receptors. As noted above, SP-A and SP-D play important functional roles in the response to RSV infection (LeVine *et al*., 1999; Ghildyal *et al*., 1999; Hickling *et al*., 1999; Lahti *et al*., 2002; Lofgren *et al*., 2002).

Children with RSV bronchiolitis show increased levels of IL-8/CXCL8 (Abu-Harb *et al*., 1999), RANTES/CCL5 (Chung and Kim, 2002), IP-10/CXCL10, monocyte chemoattractant protein (MCP)-1 (CCL2) and macrophage inflammatory protein (MIP)-1α (CCL3, an eosinophil chemoattractant) (Garofalo *et al*., 2001) in the airway. Additional studies examining RSV-infected cultured airway epithelial cells have confirmed these results (Olszewska-Pazdrak *et al*., 1998), and also added additional chemokines, most notably MIP-1β/CCL4, MIP-3α/CCL20, CXCL11, GRO-α/CXCL1 and fractalkine/CX_3CL1, a unique chemokine which may exist as either a membrane-anchored adhesion molecule or a soluble chemoattractant for T-cells and monocytes (Zhang *et al*., 2001). Thus, RSV is a potent stimulus for neutrophil, eosinophil and T-cell chemotaxis. IL-6 and IL-10 levels are also increased in the airway of infants with bronchiolitis (Sheeran *et al*., 1999).

The RSV genome encodes 10 proteins. They are non-structural (NS)-1 and 2, nucleocapsid, phosphoprotein, matrix (M)-1 and 2, small hydrophobic (SH), surface attachment glycoprotein (G), surface fusion glycoprotein (F), and RNA-dependent RNA polymerase. Binding to the respiratory epithelium occurs via interactions between the heparin-binding domains of the G glycoprotein and glycosaminoglycans on the cell surface (Martinez and Melero, 2000). RSV G glycoprotein may also interact with L-selectin (CD62L) and annexin II (Malhotra *et al*., 2003). RSV uses lipid rafts for assembly and budding (Marty *et al*., 2004).

Recent studies suggest that, in addition to terminating host cell translation and promoting viral replication, RSV proteins may play specific roles in modulating the immune response. The non-glycosylated, central, conserved region of the G glycoprotein contains a CX_3C chemokine motif capable of interacting with the fractalkine chemokine receptor, CX3CR1 (Tripp *et al*., 2001). It is, therefore, conceivable that G glycoprotein expression inhibits fractalkine-mediated chemotaxis, enhancing viral persistence. The RSV NS2 protein is

sufficient to decrease epithelial cell signal transducer and activator of transcription (STAT)-2 and type I interferon-induced gene expression (Ramaswamy *et al*., 2004). NS2 also suppresses premature apoptosis, thereby facilitating viral growth (Bitko *et al*., 2007). Finally, intranasal siRNA particles targeting NS1 have recently been shown to inhibit RSV infection, suggesting that this viral protein also modulates the immune response (Zhang *et al*., 2005).

RSV specifically targets the apical surface of ciliated epithelial cells, is shed exclusively from the apical surface, and spreads to neighbouring ciliated cells by the motion of the cilial beat (Zhang *et al*., 2002). As in the case of RV, much is known about the signal transduction pathways regulating RSV-induced chemokine expression. The RSV F glycoprotein is sufficient for cytokine expression in cultured monocytes, and CD14 and TLR4 are each required for this response (Kurt-Jones *et al*., 2000). RSV infection of respiratory epithelial cells induces increased TLR4 mRNA expression and TLR4 membrane localization, suggesting that TLR4 signalling may also play a role in RSV-induced epithelial cell responses (Monick *et al*., 2003). RSV inhibits apoptosis and induces nuclear factor (NF)-κB activity through a PI 3-kinase-dependent pathway (Thomas *et al*., 2002). Cytoskeletal reorganization during viral endocytosis activates RhoA, which is required for RSV-induced syncytium formation and filamentous virion morphology (Gower *et al*., 2005). RSV induces STAT activation and interferon regulatory factor (IRF) gene expression via a redox-sensitive pathway involving the inhibition of tyrosine phosphatase activity (Liu *et al*., 2004). IκB kinase-ε is also required (Indukuri *et al*., 2006). RSV also activates NF-κB activation and IL-8 expression via a non-canonical pathway (Jamaluddin *et al*., 1998). As with RV, RSV requires full-length positive-sense RNA for synthesis of new viral RNA, thereby requiring the formation of double-stranded intermediates. Accordingly, RSV upregulates TLR3 expression in A549 epithelial cells, and inhibition of TLR3 expression decreases RSV-induced synthesis of IP-10/CXCL10, CCL5 and IFN-β, but not IL-8/CXCL8 (Rudd *et al*., 2005; Liu *et al*., 2007). Upregulation of TLR3, in turn, is dependent on the RNA helicase RIG-1 (Liu *et al*., 2007). RIG-1 is also required for RSV-induced IFN-β, IP-10/CXCL10, CCL-5 and IFN-stimulated gene 15 expression during the early phase of infection, but not at later times (Liu *et al*., 2007).

RSV infection has also been associated with neurogenic airway inflammation (King *et al*., 2001). Unmyelinated sensory nerve fibres that innervate the lung are located below the epithelial surface. During RSV infection, these neuronal cells release pro-inflammatory neuropeptides such as substance P and neurokinin A, leading to bronchoconstriction, vasodilation of the tracheobronchial microcirculation, increased permeability of the postcapillary venules, and exudative edema of the airway mucosa. RSV proteins G and/or SH are required for this response (Tripp *et al*., 2000). Substance P expression stimulates monocytes and macrophages to release a variety of mediators including IL-1, IL-6, IL-10, IL-12, and TNF-α (Azzolina *et al*., 2003; Weinstock *et al*., 2003). Nerve growth factor (NGF) arising from infected airway epithelial cells increases the production and release of substance P from sensory neurons. Following RSV infection of F-344 rats, expression of NGF and the neurokinin 1 substance P receptor are increased (King *et al*., 2001). NGF is overexpressed in the lower airway of infants with RSV (Tortorolo *et al*., 2005). Finally, capsaicin treatment of RSV-infected rats increases leukocyte infiltration of the airway (Auais *et al*., 2003). It is also conceivable that interaction of RSV glycoprotein G or fractalkine with the neuronal fractalkine receptor could increase substance P expression (Tripp, 2004). Taken together, these data suggest that, in the context of RSV infection, sensory nerve stimulation plays a significant physiologic and immunomodulatory role.

12.8 Influenza

Influenza is a cause of bronchiolitis (Loda *et al*., 1968) and pneumonia in infants (McIntosh, 2002), community-acquired pneumonia in adults (File, 2003), and exacerbations of asthma and COPD (Johnston *et al*., 1995; Nicholson *et al*., 1993; Greenberg *et al*., 2000; Seemungal *et al*., 2001; Rohde *et al*., 2003). The influenza viruses (A, B and C) are negative-stranded RNA viruses of the *Orthomyxoviridae* family. The genome of influenza A, the most pathogenic virus, consists of eight RNA segments of variable size encoding 10 proteins. Influenza A viruses are classified according to their haemagglutinin (H1-H5) and neuraminidase (N1-N9) proteins. Viruses with haemagglutinins H1, H2 or H3 and neuraminidases N1 or N2 are endemic in humans. Influenza cells replicate in airway epithelial cells, but leukocytes are also infected.

The first stage in influenza virus entry to a host cell is recognition of terminal sialic acid on glycosylated cell surface molecules by haemagglutinin. Human influenza viruses bind to receptor molecules bearing α-2,6-linked sialic acid, while avian influenza A (H5N1) strains preferentially bind to α-2,3-linked sialic acid (Rogers and Paulson, 1983). In cultured primary airway epithelial cells, human viruses preferentially infect non-ciliated cells with predominantly 2-6-linked sialic acids, whereas avian viruses mainly infect ciliated cells with 2-3-linked sialic acids (Matrosovich *et al*., 2004). On the other hand, a study of human lower respiratory tract tissue from patients with avian influenza showed that H5N1 virus attaches predominantly to type II pneumocytes, alveolar macrophages, and non-ciliated cuboidal epithelial cells in terminal bronchioles, perhaps contributing to the severity of the pulmonary lesion (van Riel *et al*., 2006). This preference for the terminal airway and alveoli may also be a limiting factor in human-to-human transmissibility of H5N1 virus.

Influenza virus haemagglutinin concentrates in lipid raft microdomains for efficient viral fusion (Takeda *et al*., 2003). Cell entry occurs via clathrin-dependent endocytosis. Low pH in endosomes triggers the fusion of viral and endosomal membranes, liberating viral ribonucleoprotein complexes into the cytoplasm. Following viral replication, productive influenza virus infection in epithelial cells destroys host cell pre-RNAs, inhibits translation of cellular mRNAs and kills the host cells either by cytolytic or apoptotic mechanisms. Once again, virus-infected cells respond to infection by production of chemotactic, pro-inflammatory and antiviral proteins.

Epithelial cells produce IL-6, RANTES/CCL5, MCP-1/CCL2, IL-8/CXCL8 and eotaxin/CCL11 in response to influenza A virus infection (Choi and Jacoby, 1992; Adachi *et al*., 1997; Matsukura *et al*., 1996; Kawaguchi *et al*., 2001). After experimental influenza infection of human volunteers, nasal lavage fluids contain elevated levels of cytokines and chemokines including IL-6, IL-8, IL-10, TNF-α, IL-8, MIP-1α/CCL3, MIP-1β/CCL4 and MCP-1 (Hayden *et al*., 1998; Fritz *et al*., 1999; Skoner *et al*., 1999). It has been hypothesized that hyperinduction of pro-inflammatory cytokines is the cause of unusual disease severity following avian influenza infection. Expression of TNF-α, IFN-α/β, IL-1β, MCP-1, MIP-1α, IL-12 and MIP-1β are increased in H5N1-induced human macrophages compared to those infected with H1N1 (Cheung *et al*., 2002). In contrast to virus-infected monocytes and macrophages, human lung epithelial cell lines show poor induction of IFN-α/β during influenza A infection (Ronni *et al*., 1997). In lung A549 cells, influenza A virus-induced expression of IFN-α/β, IFN-λ1 and IFN-γ2 (IL-28 and 29) is dependent on pretreatment with IFN-α or TNF-α (Matikainen *et al*., 2006). It has recently been shown

that, unlike dendritic cells, airway epithelial cells require a positive-feedback mechanism involving the IFN-stimulated transcription factor IRF-7 for maximal IFN-α/β production (Prakash *et al*., 2005).

Several studies have examined the signal transduction pathways regulating influenza-induced airway epithelial cell gene expression. p38 and JNK mitogen-activated protein kinases regulate RANTES production by influenza virus-infected human bronchial epithelial cells (Kujime *et al*., 2000). In BEAS-2B airway epithelial cells, influenza A-induced IL-8 expression is inhibited by chemical inhibitors of ERK, JNK and PI 3-kinase (Guillot *et al*., 2005). As expected, IKK is a key factor in triggering influenza A virus-induced inflammatory cytokine production in airway epithelial cells (Bernasconi *et al*., 2005). In HEK293 cells, influenza virus-induced NF-κB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IKK (Flory *et al*., 2000). Together with NF-κB and IRF-3/7, the JNK effectors c-Jun and ATF-2 (transcription factors of the AP-1 family), are critical regulators of IFN-β expression in epithelial cell lines (Ludwig *et al*., 2001). Influenza A virus-induced transactivation of NF-κB and the IRF/IFN-sensitive response element in BEAS-2B cells is inhibited by a dominant-negative mutant of the TLR-3 adaptor protein TRIF, implying a role for TLR3 in this process (Guillot *et al*., 2005). However, in A549 cells, a dominant-negative form of RIG-I inhibits influenza A virus-induced IFN-β promoter activity in TNF-α-pretreated cells, and selective activation of RIG-I or IKK-ε but not TLR3 enhances IFN-β, and -λ gene expression, suggesting that RIG-I is the primary sensor of influenza A dsRNA in respiratory epithelial cells (Matikainen *et al*., 2006).

It has been postulated that the induction of apoptosis is a host defence mechanism, stopping the replication and spread of virus. Apoptosis is an essential process for the destruction of potentially harmful cells, including virus-infected cells. However, some viruses have apparently learned either to interfere with apoptosis in order to promote their own replication or to utilize apoptosis to their own advantage. In RV-infected primary airway epithelial cells, inhibition of apoptosis results in enhanced viral yield (Wark *et al*., 2005). On the other hand, in HeLa cells, apoptosis has no effect on RV14 replication and facilitates release of newly formed virus from cells (Deszcz *et al*., 2005). Influenza virus propagation is strongly impaired by inhibition of caspase 3 in MDCK cells and ectopic expression of this protein boosts replication efficiency (Wurzer *et al*., 2003). Caspase 3 activation enhanced export of viral ribonucleoprotein complexes from the nucleus, allowing formation of progeny virus particles. Thus, early induction of caspase activity may support viral propogation, in contrast to execution of the full apoptotic process, which is most likely an antiviral response.

As noted above, RSV proteins, particularly NS2, may play specific roles in modulating the immune response, including the suppression of antiviral gene expression (Ramaswamy *et al*., 2004) and premature apoptosis (Bitko *et al*., 2007). Influenza infection also interferes with the antiviral response. Blockade of the ERK signalling pathway strongly impairs influenza A growth, and active mutants of Raf-1 and MEK, upstream activators of ERK, enhance viral titres (Pleschka *et al*., 2001). Activation of ERK is required for nuclear export of viral ribonucleoprotein complexes by NS2. The viral NS1 protein limits influenza-induced activation of NF-κB and ds-RNA dependent PKR (Wang *et al*., 2000; Bergmann *et al*., 2000), as well as IFN-α/β production (Garcia-Sastre *et al*., 1998), perhaps by its ability to bind to dsRNA (Lu *et al*., 1994).

12.9 Interactions between viral infections and other stimuli

As noted above, investigators have hypothesized that RSV infection skews the immune response towards an allergic phenotype. In murine models, the timing of RSV infection appears to be critical in determining how the immune system responds. In mice infected with RSV during the allergen sensitization, infection prolongs methacholine-induced airway hyperresponsiveness (Peebles *et al*., 1999). In contrast, RSV infection before allergic sensitization decreases allergen-induced airway hyperresponsiveness, production of IL-13 in lung tissue, and lung eosinophilia (Peebles *et al*., 2001). RSV infection before and during allergen challenge decreases mucus-secreting cells and alveolitis (Barends *et al*., 2004). Taken together, these data suggest that that RSV enhances allergic disease only when the immune system has already been Th2-primed by the allergen, consistent with clinical data showing that IFN responses at birth predict RSV-induced wheeze in the first year of life (Gern *et al*., 2006). Like RSV, influenza infection before allergen airway challenge strongly suppresses allergen-induced airway eosinophilia (Wohlleben *et al*., 2003). While the relationship between viral infection and the allergic response largely reflects the interaction between virus and T-lymphocytes, it is likely that airway epithelial cell cytokine and chemokine responses to viral infection modulate this interaction.

Polymicrobial infection is an important factor in the pathogenesis of acute and chronic lung diseases. Adverse outcomes due to co-infection of viruses and bacteria have been recognized in several respiratory diseases including pneumonia, sinusitis, pertussis (whooping cough), and COPD. In a recent clinical study, mixed viral and bacterial infections, specifically rhinovirus and NTHI were shown to be associated with increased lung inflammation, decreased lung function and greater exacerbation severity in COPD patients compared to that caused by either pathogen alone (Wilkinson *et al*., 2006). Animal models of mixed viral and bacterial infections strongly support the notion that prior pulmonary infection with respiratory viruses such as influenza and RSV greatly increases the severity of bacterial infection (Peltola *et al*., 2005).

Cellular mechanisms responsible for interactions between viral and bacterial infection of the airway epithelium have recently been elucidated. Viruses can predispose the host to bacterial infection by various mechanisms including destruction of respiratory epithelium, induction of immunosuppression and increasing expression of molecules that bacteria use as receptors. Both influenza A and RSV cause damage to ciliary cells or ciliostasis, impairing airway clearance mechanisms (Giebink *et al*., 1987; Tristram *et al*., 1998). RSV infection enhances adherence of *Streptococcus pneumoniae* to human epithelial cells (Hament *et al*., 2004). Influenza A virus increases adherence and internalization of Group A *Staphylococcus aureus* by destroying respiratory epithelium, thus exposing the basement membrane (Okamoto *et al*., 2003). The specific level of their neuraminidase activity correlates with the capacity of influenza infection to support secondary bacterial pneumonia (Peltola *et al*., 2005).

In addition, pre-existing chronic bacterial infection in the lower airway may render the host susceptible to viral infection. We have recently demonstrated that the prior infection with non-typeable *Hemophilus influenzae* potentiates pro-inflammatory responses of well-differentiated primary airway epithelial cells to RV infection. The observed increased responses were partly dependent on the increased expression of ICAM-1 and TLR3 (Sajjan *et al*., 2006). We also noted increased expression of TLR3 in lower airway of COPD patients with chronic bacterial colonization of the airways (U. Sajjan, M. Hershenson, D. Arenberg and F. Martinez, unpublished data).

Air pollutants such as cigarette smoke, particulate matter, ozone and nitrogen dioxide activate the innate defences of the lung, suggesting the possibility of interactions between air pollution and viral infection. Mice exposed to diesel engine emissions manifest a greater influenza disease burden and lower interferon levels (Hahon *et al*., 1985). Prior diesel emission exposure also increases RSV gene expression and viral-induced cytokine expression, peribronchial inflammation and mucous cell metaplasia, while decreasing levels of Clara cell secretory protein, pro-SP-B and SP-A (Harrod *et al*., 2003). Ultrafine carbon black particles enhance RSV-induced airway reactivity, pulmonary inflammation, and chemokine expression in mice (Lambert *et al*., 2003). Recently, it has been shown that diesel exhaust particles increase rat lung epithelial cell expression of both ICAM-1 and LDL (Ito *et al*., 2006), each of which serve as receptors for RV. In patients with asthma, exacerbations related to cold symptoms are associated with higher levels of sulfur dioxide and nitrogen oxides from March to November in comparison with asthma exacerbations without cold symptoms (Tarlo *et al*., 2001). High exposure to nitrogen dioxide the week before the start of a respiratory viral infection has been associated with an increase in the severity of a resulting asthma exacerbation (Chauhan *et al*., 2003). Thus, air pollution may exacerbate viral-induced disease by activating chemokine expression, increasing viral infectivity, blunting airway defences and inhibiting viral clearance.

References

Abu-Harb, M., Bell, F., Finn, A., Rao, W. H., Nixon, L., Shale, D. & Everard, M. L. (1999) IL-8 and neutrophil elastase levels in the respiratory tract of infants with RSV bronchiolitis. *Eur. Respir. J*., **14**, 139–143.

Adachi, M., Matsukura, S., Tokunaga, H. & Kokubu, F. (1997) Expression of cytokines on human bronchial epithelial cells induced by influenza virus A. *Int. Arch. Allergy Immunol*., **113**, 307–311.

Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y. M., Dietzschold, B. & Maeda, H. (1996) Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc. Natl Acad. Sci. USA*, **93**, 2448–2453.

Ali-Ahmad, D., Bonville, C. A., Rosenberg, H. F. & Domachowske, J. B. (2003) Replication of respiratory syncytial virus is inhibited in target cells generating nitric oxide in situ. *Front. Biosci*., **8**, 48–53.

Auais, A., Adkins, B., Napchan, G. & Piedimonte, G. (2003) Immunomodulatory effects of sensory nerves during respiratory syncytial virus infection in rats. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **285**, L105–L113.

Azzolina, A., Bongiovanni, A. & Lampiasi, N. (2003) Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells. *Biochim. Biophys. Acta*, **1643**, 75–83.

Barends, M., Van Oosten, M., De Rond, C. G., Dormans, J. A., Osterhaus, A. D., Neijens, H. J. & Kimman, T. G. (2004) Timing of infection and prior immunization with respiratory syncytial virus (RSV) in RSV-enhanced allergic inflammation. *J. Infect. Dis*., **189**, 1866–1872.

Belvisi, M. G., David Stretton, C., Yacoub, M. & Barnes, P. J. (1992) Nitric oxide is the endogenous neurotransmitter of bronchodilator nerves in humans. *Eur. J. Pharmacol*., **210**, 221–222.

Bentley, J. K., Newcomb, D. C., Goldsmith, A. M., Jia, Y., Sajjan, U. S. & Hershenson, M. B. (2007) Rhinovirus activates IL-8 expression via a Src/p110β PI 3-kinase/Akt pathway in human airway epithelial cells. *J. Virol*., **81**, 1186–1194.

Bergmann, M., Garcia-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P. & Muster, T. (2000) Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *J. Virol*., **74**, 6203–6206.

Bernasconi, D., Amici, C., La Frazia, S., Ianaro, A. & Santoro, M. G. (2005) The IkappaB kinase is a key factor in triggering influenza A virus-induced inflammatory cytokine production in airway epithelial cells. *J. Biol. Chem.*, **280**, 24127–24134.

Bhowmik, A., Seemungal, T. A. R., Sapsford, R. J. & Wedzicha, J. A. (2000) Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax*, **55**, 114–120.

Biragyn, A., Ruffini, P. A., Leifer, C. A., Klyushnenkova, E., Shakhov, A., Chertov, O., Shirakawa, A. K., Farber, J. M., Segal, D. M., Oppenheim, J. J. & Kwak, L. W. (2002) Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science*, **298**, 1025–1029.

Bitko, V., Shulyayeva, O., Mazumder, B., Musiyenko, A., Ramaswamy, M., Look, D. C. & Barik, S. (2007) The nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by a NF-kappa B-dependent, interferon-independent mechanism and facilitate virus growth. *J. Virol.*, **81**, 1786–1795.

Bossios, A., Psarras, S., Gourgiotis, D., Skevaki, C. L., Constantopoulos, A. G., Saxoni-Papageorgiou, P. & Papadopoulos, N. G. (2005) Rhinovirus infection induces cytotoxicity and delays wound healing in bronchial epithelial cells. *Respir. Res.*, **6**, 114.

Brooks, G. D., Buchta, K. A., Swenson, C. A., Gern, J. E. & Busse, W. W. (2003) Rhinovirus-induced interferon-{gamma} and airway responsiveness in asthma. *Am. J. Respir. Crit. Care Med.*, **168**, 1091–1094.

Chauhan, A. J., Inskip, H. M., Linaker, C. H., Smith, S., Schreiber, J., Johnston, S. L. & Holgate, S. T. (2003) Personal exposure to nitrogen dioxide (NO2) and the severity of virus-induced asthma in children. *Lancet*, **361**, 1939–1944.

Chen, Y., Hamati, E., Lee, P.-K., Lee, W.-M., Wachi, S., Schnurr, D., Yagi, S., Dolganov, G., Boushey, H., Avila, P. & Wu, R. (2006) Rhinovirus induces airway epithelial gene expression through double-stranded RNA and IFN-dependent pathways. *Am. J. Respir. Cell. Mol. Biol.*, **34**, 192–203.

Cheung, C. Y., Poon, L. L. M., Lau, A. S., Luk, W., Lau, Y. L., Shortridge, K. F., Gordon, S., Guan, Y. & Peiris, J. S. M. (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet*, **360**, 1831–1837.

Choi, A. M. & Jacoby, D. B. (1992) Influenza virus A infection induces interleukin-8 gene expression in human airway epithelial cells. *FEBS Lett.*, **309**, 327–329.

Chung, H. L. & Kim, S. G. (2002) RANTES may be predictive of later recurrent wheezing after respiratory syncitial virus bronchiolitis in infants. *Am. Allergy Asthma Immunol.*, **88**, 463–467.

Contoli, M., Message, S. D., Laza-Stanca, V., Edwards, M. R., Wark, P. A., Bartlett, N. W., Kebadze, T., Mallia, P., Stanciu, L. A., Parker, H. L., Slater, L., Lewis-Antes, A., Kon, O. M., Holgate, S. T., Davies, D. E., Kotenko, S. V., Papi, A. & Johnston, S. L. (2006) Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat. Med.*, **12**, 1023–1026.

De Kluijver, J., Grunberg, K., Pons, D., De Klerk, E. P., Dick, C. R., Sterk, P. J. & Hiemstra, P. S. (2003) Interleukin-1beta and interleukin-1ra levels in nasal lavages during experimental rhinovirus infection in asthmatic and non-asthmatic subjects. *Clin. Exp. Allergy*, **33**, 1415–1418.

Deszcz, L., Gaudernak, E., Kuechler, E. & Seipelt, J. (2005) Apoptotic events induced by human rhinovirus infection. *J. Gen. Virol.*, **86**, 1379–1389.

DeTulleo, L. & Kirchhausen, T. (1998) The clathrin endocytic pathway in viral infection. *EMBO J.*, **17**, 4585–4593.

Eller, J., Ede, A., Schaberg, T., Niederman, M., Mauch, H. & Lode, H. (1998) Infective exacerbations of chronic bronchitis: relation between bacteriologic etiology and lung function. *Chest*, **113**, 1542–1548.

Falsey, A. R. & Walsh, E. E. (2006) Viral pneumonia in older adults. *Clin. Infect. Dis.*, **42**, 518–524.

File, T. M. (2003) Community-acquired pneumonia. *Lancet*, **362**, 1991–2001.

Flory, E., Kunz, M., Scheller, C., Jassoy, C., Stauber, R., Rapp, U. R. & Ludwig, S. (2000) Influenza virus-induced NF-kappa B-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of Ikappa B Kinase. *J. Biol. Chem*., **275**, 8307–8314.

Fraenkel, D. J., Bardin, P. G., Sanderson, G., Lampe, F., Johnston, S. L. & Holgate, S. T. (1995) Lower airways inflammation during rhinovirus colds in normal and in asthmatic subjects. *Am. J. Respir. Crit. Care Med*., **151**, 879–886.

Freymouth, F., Vabret, A. & Legrand, L. (2003) Presence of the new human metapneumovirus in French children with bronchiolitis. *Pediatr. Infect. Dis. J*., **22**, 92–94.

Fritz, R. S., Hayden, F. G., Calfee, D. P., Cass, L. M., Peng, A. W., Alvord, W. G., Strober, W. & Straus, S. E. (1999) Nasal cytokine and chemokine responses in experimental influenza A virus infection: results of a placebo-controlled trial of intravenous zanamivir treatment. *J. Infect. Dis*., **180**, 586–593.

Funkhouser, A. W., Kang, J. A., Tan, A., Li, J., Zhou, L., Abe, M. K., Solway, J. & Hershenson, M. B. (2004) Rhinovirus 16 3C protease induces interleukin-8 and granulocyte-macrophage colony-stimulating factor expression in human bronchial epithelial cells. *Pediatr. Res*., **55**, 13–18.

Garcia-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P. & Muster, T. (1998) Influenza A virus lacking the NS1 Gene replicates in interferon-deficient systems. *Virology*, **252**, 324–330.

Garofalo, R. P., Patti, J., Hintz, K. A., Hill, V., Ogra, P. L. & Welliver, R. C. (2001) Macrophage inflammatory protein-1alpha (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. *J. Infect. Dis*., **184**, 393–399.

Gern, J. E., Galagan, D. M., Jarjour, N. N., Dick, E. C. & Busse, W. W. (1997) Detection of rhinovirus RNA in lower airway cells during experimentally induced infection. *Am. J. Respir. Crit. Care Med*., **155**, 1159–1161.

Gern, J. E., French, D. A., Grindle, K. A., Brockman-Schneider, R. A., Konno, S.-I. & Busse, W. W. (2003) Double-stranded RNA induces the synthesis of specific chemokines by bronchial epithelial cells. *Am. J. Respir. Cell. Mol. Biol*., **28**, 731–737.

Gern, J. E., Brooks, G. D., Meyer, P., Chang, A., Shen, K., Evans, M. D., Tisler, C., Dasilva, D., Roberg, K. A., Mikus, L. D., Rosenthal, L. A., Kirk, C. J., Shult, P. A., Bhattacharya, A., Li, Z., Gangnon, R. & Lemanske, R. F. (2006) Bidirectional interactions between viral respiratory illnesses and cytokine responses in the first year of life. *J. Allergy Clin. Immunol*., **117**, 72–78.

Ghildyal, R., Hartley, C., Varrasso, A., Meanger, J., Voelker, D. R., Anders, E. M. & Mills, J. (1999) Surfactant protein A binds to the fusion glycoprotein of respiratory syncytial virus and neutralizes virion infectivity. *J. Infect. Dis*.., **180**, 2009–2013.

Giebink, G. S., Ripley, M. L. & Wright, P. F. (1987) Eustachian tube histopathology during experimental influenza A virus infection in the chinchilla. *Ann. Otol. Rhinol. Laryngol*., **96**, 199–206.

Glezen, W. P., Taber, L. H. & Frank, A. L. (1986) Risk of primary infection and reinfection with respiratory syncytial virus. *Am. J. Dis. Child*, **140**, 543–546.

Gower, T. L., Pastey, M. K., Peeples, M. E., Collins, P. L., McCurdy, L. H., Hart, T. K., Guth, A., Johnson, T. R. & Graham, B. S. (2005) RhoA signaling is required for respiratory syncytial virus-induced syncytium formation and filamentous virion morphology. *J. Virol*., **79**, 5326–5336.

Graham, B. S., Bunton, L. A., Wright, P. F. & Karzon, D. T. (1991) Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J. Clin. Invest*., **88**, 1026–1033.

Grassme, H., Riehle, A., Wilker, B. & Gulbins, E. (2005) Rhinoviruses infect human epithelial cells via ceramide-enriched membrane platforms. *J. Biol. Chem*., **280**, 26256–26262.

Greenberg, S. B., Allen, M., Wilson, J. & Atmar, R. L. (2000) Respiratory viral infections in adults with and without chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med*., **162**, 167–173.

Griego, S. D., Weston, C. B., Adams, J. L., Tal-Singer, R. & Dillon, S. B. (2000) Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J. Immunol*., **165**, 5211–5220.

Grunberg, K., Smits, Hermelijn H., Timmers, Mieke C., De Klerk, Erik P. A., Dolhain, Radboud J. E. M., Dick, Elliot C., Hiemstra, Pieter S. & Sterk, Peter J. (1997a) Experimental rhinovirus 16 infection. Effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am. J. Respir. Crit. Care Med*., **156**, 609–616.

Grunberg, K., Timmers, M. C., Smits, H. H., De Klerk, E. P., Dick, E. C., Spaan, W. J., Hiemstra, P. S. & Sterk, P. J. (1997b) Effect of experimental rhinovirus 16 colds on airway hyperresponsiveness to histamine and interleukin-8 in nasal lavage in asthmatic subjects in vivo. *Clin. Exp. Allergy*, **27**, 36–45.

Grunberg, K., Sharon, R. F., Hiltermann, T. J., Brahim, J. J., Dick, E. C., Sterk, P. J. & Van Krieken, J. H. (2000) Experimental rhinovirus 16 infection increases intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment. *Clin. Exp. Allergy*, **30**, 1015–1023.

Grunberg, K., Sharon, R. F., Sont, J. K., In't Veen, J. C. C. M., Van Schadewijk, W. A. A. M., De Klerk, E. P. A., Dick, C. R., Van Krieken, J. H. J. M. & Sterk, P. J. (2001) Rhinovirus-induced airway inflammation in asthma: effect of treatment with inhaled corticosteroids before and during experimental infection. *Am. J. Respir. Crit. Care Med*., **164**, 1816–1822.

Grunert, H. P., Wolf, K. U., Langner, K. D., Sawitzky, D., Habermehl, K. O. & Zeichhardt, H. (1997) Internalization of human rhinovirus 14 into HeLa and ICAM-1-transfected BHK cells. *Med. Microbiol. Immunol. (Berl.)*, **186**, 1–9.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M. & Si-Tahar, M. (2005) Involvement of Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem*., **280**, 5571–5580.

Guo, F. H., Comhair, S. A. A., Zheng, S., Dweik, R. A., Eissa, N. T., Thomassen, M. J., Calhoun, W. & Erzurum, S. C. (2000) Molecular mechanisms of increased nitric oxide (NO) in asthma: Evidence for transcriptional and post-translational regulation of NO synthesis. *J. Immunol*., **164**, 5970–5980.

Hahon, N., Booth, J. A., Green, F. & Lewis, T. R. (1985) Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. *Environ. Res*., **37**, 44–60.

Hakonarson, H., Maskeri, N., Carter, C., Hodinka, R. L., Campbell, D. & Grunstein, M. M. (1998) Mechanism of rhinovirus-induced changes in airway smooth muscle responsiveness. *J. Clin. Invest*., **102**, 1732–1741.

Halperin, S. A., Eggleston, P. A., Hendley, J. O., Suratt, P. M., Groschel, D. H. & Gwaltney, J. M. (1983) Pathogenesis of lower respiratory tract symptoms in experimental rhinovirus infection. *Am. Rev. Respir. Dis*., **128**, 806–810.

Hament, J. M., Aerts, P. C., Fleer, A., Van Dijk, H., Harmsen, T., Kimpen, J. L. & Wolfs, T. F. (2004) Enhanced adherence of *Streptococcus pneumoniae* to human epithelial cells infected with respiratory syncytial virus. *Pediatr. Res*., **55**, 972–978.

Harrod, K. S., Jaramillo, R. J., Rosenberger, C. L., Wang, S.-Z., Berger, J. A., McDonald, J. D. & Reed, M. D. (2003) Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. *Am. J. Respir. Cell. Mol. Biol*., **28**, 451–463.

Hayden, F. G., Fritz, R. S., Lobo, M. C., Alvord, W. G., Strober, W. & Straus, S. E. (1998) Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defence. *J. Clin. Invest*., **101**, 643–649.

Hickling, T. P., Bright, H., Wing, K., Gower, D., Martin, S. L., Sim, R. B. & Malhotra, R. (1999) A recombinant trimeric surfactant protein D carbohydrate recognition domain inhibits respiratory syncytial virus infection in vitro and in vivo. *Eur. J. Immunol*., **29**, 3478–3484.

Hickling, T. P., Clark, H., Malhotra, R. & Sim, R. B. (2004) Collectins and their role in lung immunity. *J. Leukoc. Biol*., **75**, 27–33.

Holman, R. C., Shay, D. K. & Curns, A. T. (2003) Risk factors for bronchiolitis-associated deaths among infants in the United States. *Pediatr. Infect. Dis. J*., **22**, 483–490.

Indukuri, H., Castro, S. M., Liao, S. M., Feeney, L. A., Dorsch, M., Coyle, A. J., Garofalo, R. P., Brasier, A. R. & Casola, A. (2006) Ikkepsilon regulates viral-induced interferon regulatory factor-3 activation via a redox-sensitive pathway. *Virology*, **353**, 155–165.

Ito, T., Okumura, H., Tsukue, N., Kobayashi, T., Honda, K. & Sekizawa, K. (2006) Effect of diesel exhaust particles on mRNA expression of viral and bacterial receptors in rat lung epithelial L2 cells. *Toxicol. Lett*., **165**, 66–70.

Jamaluddin, M., Casola, A., Garofalo, R. P., Han, Y., Elliott, T., Ogra, P. L. & Brasier, A. R. (1998) The major component of IkappaBalpha proteolysis occurs independently of the proteasome pathway in respiratory syncytial virus-infected pulmonary epithelial cells. *J. Virol*., **72**, 4849–4857.

Johnston, S. L., Pattemore, P. K., Sanderson, G., Smith, S., Lampe, F., Josephs, L., Symington, P., O'Toole, S., Myint, S. H., Tyrrell, D. A. & Holgate, S. T. (1995) Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *Brit. Med. J*., **310**, 1225–1229.

Kaiser, L., Aubert, J.-D., Pache, J.-C., Deffernez, C., Rochat, T., Garbino, J., Wunderli, W., Meylan, P., Yerly, S., Perrin, L., Letovanec, I., Nicod, L., Tapparel, C. & Soccal, P. M. (2006) Chronic rhinoviral infection in lung transplant recipients. *Am. J. Respir. Crit. Care Med*., **174**, 1392–1399.

Karupiah, G., Chen, J.-H., Mahalingam, S., Nathan, C. F. & Macmicking, J. D. (1998) Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J. Exp. Med*., **188**, 1541–1546.

Kaul, P., Biagioli, M. C., Singh, I. & Turner, R. B. (2000) Rhinovirus-induced oxidative stress and interleukin-8 elaboration involves p47-phox but is independent of attachment to intercellular adhesion molecule-1 and viral replication. *J. Infect. Dis*., **181**, 1885–1890.

Kawaguchi, M., Kokubu, F., Kuga, H., Tomita, T., Matsukura, S., Suzaki, H., Huang, S. K. & M, A. (2001) Influenza virus A stimulates expression of eotaxin by nasal epithelial cells. *Clin. Exp. Allergy*, **31**, 873–80.

Keyaerts, E., Vijgen, L., Chen, L., Maes, P., Hedenstierna, G. & Van Ranst, M. (2004) Inhibition of SARS-coronavirus infection in vitro by *S*-nitroso-*N*-acetylpenicillamine, a nitric oxide donor compound. *Int. J. Infect. Dis*., **8**, 223–226.

Kim, H. W., Arrobio, J. O. & Brandt, D. C. (1973) Epidemiology or respiratory syncytial virus infection in Washington, DC. *Am. J. Epidemiol*., **98**, 216–225.

King, K. A., Hu, C., Rodriguez, M. M., Romaguera, R., Jiang, X. & Piedimonte, G. (2001) Exaggerated neurogenic inflammation and substance P receptor upregulation in RSV-infected weanling rats. *Am. J. Respir. Cell. Mol. Biol*., **24**, 101–107.

Kling, S., Donninger, H., Williams, Z., Vermeulen, J., Weinberg, E., Latiff, K., Ghildyal, R. & Bardin, P. (2005) Persistence of rhinovirus RNA after asthma exacerbation in children. *Clin. Exp. Allergy*, **35**, 672–678.

Korppi, M., Kotaniemi-Syrjanen, A. & Waris, M. (2004) Rhinovirus-associated wheezing in infancy: comparison with respiratory syncytial virus bronchiolitis. *Pediatr. Infect. Dis. J*., **23**, 995–999.

Kujime, K., Hashimoto, S., Gon, Y., Shimizu, K. & Horie, T. (2000) p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. *J. Immunol*., **164**, 3222–3228.

Kurt-Jones, E. A., Popova, L., Kwinn, L., Haynes, L. M., Jones, L. P., Tripp, R. A., Walsh, E. E., Freeman, M. W., Golenbock, D. T., Anderson, L. J. & Finberg, R. W. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immunol*., **1**, 398–401.

Lahti, M., Lofgren, J., Marttila, R., Renko, M., Klaavuniemi, T., Haataja, R., Ramet, M. & Hallman, M. (2002) Surfactant protein D gene polymorphism associated with severe respiratory syncytial virus infection. *Pediatr. Res*., **51**, 696–699.

Lambert, A. L., Mangum, J. B., Delorme, M. P. & Everitt, J. I. (2003) Ultrafine carbon black particles enhance respiratory syncytial virus-induced airway reactivity, pulmonary inflammation, and chemokine expression. *Toxicol. Sci*., **72**, 339–346.

Lawson, C., Ainsworth, M., Yacoub, M. & Rose, M. (1999) Ligation of ICAM-1 on endothelial cells leads to expression of VCAM-1 via a nuclear factor-kappa B-independent mechanism. *J. Immunol*., **162**, 2990–2996.

Leader, S. & Kohlhase, K. (2003) Recent trends in severe respiratory syncytial virus (RSV) among US infants, 1997 to 2000. *J. Pediatr*., **143**, S127-S312.

Lemanske, R. F., Jackson, D. J., Gangnon, R. E., Evans, M. D., Li, Z., Shult, P. A., Kirk, C. J., Reisdorf, E., Roberg, K. A., Anderson, E. L., Carlson-Dakes, K. T., Adler, K. J., Gilbertson-White, S., Pappas, T. E., Dasilva, D. F., Tisler, C. J. & Gern, J. E. (2005) Rhinovirus illnesses during infancy predict subsequent childhood wheezing. *J. Allergy Clin. Immunol*., **116**, 571–577.

Levine, A. M., Gwozdz, J., Stark, J., Bruno, M., Whitsett, J. & Korfhagen, T. (1999) Surfactant protein-A enhances respiratory syncytial virus clearance in vivo. *J. Clin. Invest*., **103**, 1015–21.

Li, D., Shirakami, G., Zhan, X. & Johns, R. A. (2000) Regulation of ciliary beat frequency by the nitric oxide-cyclic guanosine monophosphate signaling pathway in rat airway epithelial cells. *Am. J. Respir. Cell. Mol. Biol*., **23**, 175–181.

Liu, P., Jamaluddin, M., Li, K., Garofalo, R. P., Casola, A. & Brasier, A. R. (2007) Retinoic acid inducible gene-I mediates early anti-viral response and Toll like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol*.. **81**, 1401–1411.

Liu, T., Castro, S., Brasier, A. R., Jamaluddin, M., Garofalo, R. P. & Casola, A. (2004) Reactive oxygen species mediate virus-induced STAT activation: role of tyrosine phosphatases. *J. Biol. Chem*., **279**, 2461–2469.

Loda, F. A., Clyde, W. A. J. & Glezen, W. P. (1968) Studies on the role of viruses, bacteria, and *M. pneumoniae* as causes of lower respiratory tract infections in children. *J. Pediatr*., **72**, 161–176.

Lofgren, J., Ramet, M., Renko, M., Marttila, R. & Hallman, M. (2002) Association between surfactant protein A gene locus and severe respiratory syncytial virus infection in infants. *J. Infect. Dis*., **185**, 283–289.

Lu, Y., Qian, X. Y. & Krug, R. M. (1994) The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. *Genes Dev*., **8**, 1817–1828.

Ludwig, S., Ehrhardt, C., Neumeier, E. R., Kracht, M., Rapp, U. R. & Pleschka, S. (2001) Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway. *J. Biol. Chem*., **276**, 10990–10998.

Malhotra, R., Haurum, J. S., Thiel, S. & Sim, R. B. (1994) Binding of human collectins (SP-A and MBP) to influenza virus. *Biochem. J*., **304**, 455–461.

Malhotra, R., Ward, M., Bright, H., Priest, R., Foster, M. R., Hurle, M., Blair, E. & Bird, M. (2003) Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. *Microbes Infect*., **5**, 123–133.

Martinez, F. D., Morgan, W. J., Wright, A. L., Holberg, C. J. & Taussig, L. M. (1988) Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. *N. Engl. J. Med*., **319**, 1112–1117.

Martinez, I. & Melero, J. A. (2000) Binding of human respiratory syncytial virus to cells: implication of sulfated cell surface proteoglycans. *J. Gen. Virol*., **81**, 2715–2722.

Marty, A., Meanger, J., Mills, J., Shields, B. & Ghildyal, R. (2004) Association of matrix protein of respiratory syncytial virus with the host cell membrane of infected cells. *Arch. Virol*., **149**, 199–210.

Matikainen, S., Siren, J., Tissari, J., Veckman, V., Pirhonen, J., Severa, M., Sun, Q., Lin, R., Meri, S., Uze, G., Hiscott, J. & Julkunen, I. (2006) Tumor necrosis factor alpha enhances influenza A

virus-induced expression of antiviral cytokines by activating RIG-I gene expression. *J. Virol*., **80**, 3515–3522.

Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A. & Klenk, H. D. (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc. Natl Acad. Sci. USA*, **101**, 4620–4624.

Matsukura, S., Kokubu, F., Noda, H., Tokunaga, H. & Adachi, M. (1996) Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. *J. Allergy Clin. Immunol*., **98**, 1080–1087.

Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A. & Seya, T. (2003) Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol*., **171**, 3154–3162.

McFadden, E. R., Pichurko, B. M., Bowman, H. F., Ingenito, E., Burns, S., Dowling, N. & Solway, J. (1985) Thermal mapping of the airways in humans. *J. Appl. Physiol*., **58**, 564–570.

McIntosh, K. (2002) Community-acquired pneumonia. *N. Engl. J. Med*., **346**, 429–437.

Monick, M. M., Yarovinsky, T. O., Powers, L. S., Butler, N. S., Carter, A. B., Gudmundsson, G. & Hunninghake, G. W. (2003) Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J. Biol. Chem*., **278**, 53035–53044.

Mosser, A. G., Vrtis, R., Burchell, L., Lee, W.-M., Dick, C. R., Weisshaar, E., Bock, D., Swenson, C. A., Cornwell, R. D., Meyer, K. C., Jarjour, N. N., Busse, W. W. & Gern, J. E. (2005) Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am. J. Respir. Crit. Care Med*., **171**, 645–651.

Newcomb, D. C., Sajjan, U., Nanua, S., Jia, Y., Goldsmith, A. M., Bentley, J. K. & Hershenson, M. B. (2005) Phosphatidylinositol 3-kinase is required for rhinovirus-induced airway epithelial cell interleukin-8 expression. *J. Biol. Chem*., **280**, 36952–36961.

Newcomb, D. C., Sajjan, U. S., Nagarkar, D. R., Goldsmith, A. M., Bentley, J. K. & Hershenson, M. B. (2007) Cooperative effects of rhinovirus and TNF-α on airway epithelial cell chemokine expression. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **293**, L1021–L1028.

Nicholson, K. G., Kent, J. & Ireland, D. C. (1993) Respiratory viruses and exacerbations of asthma in adults. *Brit. Med. J*., **307**, 982–986.

Norzila, M. Z., Fakes, K., Henry, R. L., Simpson, J. & Gibson, P. G. (2000) Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am. J. Respir. Crit. Care Med*., **161**, 769–774.

Okamoto, S., Kawabata, S., Nakagawa, I., Okuno, Y., Goto, T., Sano, K. & Hamada, S. (2003) Influenza A virus-infected hosts boost an invasive type of streptococcus pyogenes infection in mice. *J. Virol*., **77**, 4104–4112.

Olszewska-Pazdrak, B., Casola, A., Saito, T., Alam, R., Crowe, S. E., Mei, F., Ogra, P. L. & Garofalo, R. P. (1998) Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. *J. Virol*., **72**, 4756–4764.

Ordonez, C. L., Shaughnessy, T. E., Matthay, M. A. & Fahy, J. V. (2000) Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma clinical and biologic significance. *Am. J. Respir. Crit. Care Med*., **161**, 1185–1190.

Papadopoulos, N. G., Stanciu, L. A., Papi, A., Holgate, S. T. & Johnston, S. L. (2002) A defective type 1 response to rhinovirus in atopic asthma. *Thorax*, 57, 328–32.

Papi, A. & Johnston, S. L. (1999) Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappaB-mediated transcription. *J. Biol. Chem*., **274**, 9707–9720.

Peebles, R. S., Sheller, J. R., Johnson, J. E., Mitchell, D. B. & Graham, B. S. (1999) Respiratory syncytial virus infection prolongs methacholine-induced airway hyperresponsiveness in ovalbumin-sensitized mice. *J. Med. Virol*., **57**, 186–192.

Peebles, R. S., Hashimoto, K., Collins, R. D., Jarzecka, K., Furlong, J., Mitchell, D. B., Sheller, J. R. & Graham, B. S. (2001) Immune interaction between respiratory syncytial virus infection and allergen sensitization critically depends on timing of challenges. *J. Infect. Dis*., **184**, 1374–1379.

Peltola, V. T., Murti, K. G. & McCullers, J. A. (2005) Influenza virus neuraminidase contributes to secondary bacterial pneumonia. *J. Infect. Dis*., **192**, 249–257.

Pizzichini, M. M., Pizzichini, E., Efthimiadis, A., Chauhan, A. J., Johnston, S. L., Hussack, P., Mahony, J., Dolovich, J. & Hargreave, F. E. (1998) Asthma and natural colds. Inflammatory indices in induced sputum: a feasibility study. *Am. J. Respir. Crit. Care Med*., **158**, 1178–1184.

Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rapp, U. R. & Ludwig, S. (2001) Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. *Nat. Cell Biol*., **3**, 301–305.

Prakash, A., Smith, E., Lee, C.-K. & Levy, D. E. (2005) Tissue-specific positive feedback requirements for production of type I interferon following virus infection. *J. Biol. Chem*., **280**, 18651–18657.

Proud, D., Sanders, S. P. & Wiehler, S. (2004) Human rhinovirus infection induces airway epithelial cell production of human β-defensin 2 both in vitro and in vivo. *J. Immunol*., **172**, 4637–4645.

Qiu, Y., Zhu, J., Bandi, V., Atmar, R. L., Hattotuwa, K., Guntupalli, K. K. & Jeffery, P. K. (2003) Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med*., **168**, 968–975.

Ramaswamy, M., Shi, L., Monick, M. M., Hunninghake, G. W. & Look, D. C. (2004) Specific inhibition of type I interferon signal transduction by respiratory syncytial virus. *Am. J. Respir. Cell. Mol. Biol*., **30**, 893–900.

Reading, P. C., Morey, L. S., Crouch, E. C. & Anders, E. M. (1997) Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice. *J. Virol*., **71**, 8204–8212.

Rogers, G. N. & Paulson, J. C. (1983) Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology*, **127**, 361–73.

Rohde, G., Wiethege, A., Borg, I., Kauth, M., Bauer, T. T., Gillissen, A., Bufe, A. & Schultze-Werninghaus, G. (2003) Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalization: a case-control study. *Thorax*, **58**, 37–42.

Ronni, T., Matikainen, S., Sareneva, T., Melen, K., Pirhonen, J., Keskinen, P. & Julkunen, I. (1997) Regulation of IFN-alpha/beta, MxA, 2′, 5′-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells. *J. Immunol*., **158**, 2363–2374.

Rudd, B. D., Burstein, E., Duckett, C. S., Li, X. & Lukacs, N. W. (2005) Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. *J. Virol*., **79**, 3350–3357.

Sajjan, U. S., Jia, Y., Newcomb, D. C., Bentley, J. K., Lukacs, N. W., Lipuma, J. J. & Hershenson, M. B. (2006) *H. influenzae* potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. *FASEB J*., **20**, 2121–2123.

Sanders, S. P., Siekierski, E. S., Porter, J. D., Richards, S. M. & Proud, D. (1998) Nitric oxide inhibits rhinovirus-induced cytokine production and viral replication in a human respiratory epithelial cell line. *J. Virol*., **72**, 934–942.

Sanders, S. P., Siekierski, E. S., Richards, S. M., Porter, J. D., Imani, F. & Proud, D. (2001) Rhinovirus infection induces expression of type 2 nitric oxide synthase in human respiratory epithelial cells in vitro and in vivo. *J. Allergy Clin. Immunol*., **107**, 235–243.

Schroth, M. K., Grimm, E., Frindt, P., Galagan, D. M., Konno, S.-I., Love, R. & Gern, J. E. (1999) Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am. J. Respir. Cell. Mol. Biol*., **20**, 1220–1228.

Schutte, B. C. & McCray, P. B. (2002) β-defensins in lung host defense. *Ann. Rev. Physiol*., **64**, 709–748.

Seemungal, T., Harper-Owen, R., Bhowmik, A., Moric, I., Sanderson, G., Message, S., MacCallum, P., Meade, T. W., Jeffries, D. J., Johnston, S. L. & Wedzicha, J. A. (2001) Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med*., **164**, 1618–1623.

Sethi, S. K., Bianco, A., Allen, J. T., Knight, R. A. & Spiteri, M. A. (1997) Interferon-gamma (IFN-gamma) down-regulates the rhinovirus-induced expression of intercellular adhesion molecule-1 (ICAM-1) on human airway epithelial cells. *Clin. Exp. Immunol*., **110**, 362–369.

Sethi, S., Evans, N., Grant, B. J. B. & Murphy, T. F. (2002) New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *N. Engl. J. Med*., **347**, 465–471.

Sha, Q., Truong-Tran, A. Q., Plitt, J. R., Beck, L. A. & Schleimer, R. P. (2004) Activation of airway epithelial cells by Toll-like receptor agonists. *Am. J. Respir. Cell. Mol. Biol*., **31**, 358–364.

Sheeran, P., Jafri, H., Carubelli, C., Saavedra, J., Johnson, C., Krisher, K., Sanchez, P. J. & Ramilo, O. (1999) Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. *Pediatr. Infect. Dis. J*., **18**, 115–122.

Sigurs, N., Gustafsson, P. M., Bjarnason, R., Lundberg, F., Schmidt, S., Sigurbergsson, F. & Kjellman, B. (2005) Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am. J. Respir. Crit. Care Med*., **171**, 137–141.

Skoner, D. P., Gentile, D. A., Patel, A. & Doyle, W. J. (1999) Evidence for cytokine mediation of disease expression in adults experimentally infected with influenza A virus. *J. Infect. Dis*., **180**, 10–14.

Spurrell, J. C., Wiehler, S., Zaheer, R. S., Sanders, S. P. & Proud, D. (2005) Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **289**, L85-L95.

Stein, R. T., Sherrill, D., Morgan, W. J., Holberg, C. J., Halonen, M., Taussig, L. M., Wright, A. L. & Martinez, F. D. (1999) Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet*, **354**, 541–545.

Subauste, M. C., Jacoby, D. B., Richards, S. M. & Proud, D. (1995) Infection of a human respiratory epithelial cell line with rhinovirus. Induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J. Clin. Invest*., **96**, 549–557.

Suzuki, T., Yamaya, M., Kamanaka, M., Jia, Y. X., Nakayama, K., Hosoda, M., Yamada, N., Nishimura, H., Sekizawa, K. & Sasaki, H. (2001a) Type 2 rhinovirus infection of cultured human tracheal epithelial cells: role of LDL receptor. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **280**, L409–L420.

Suzuki, T., Yamaya, M., Sekizawa, K., Hosoda, M., Yamada, N., Ishizuka, S., Nakayama, K., Yanai, M., Numazaki, Y. & Sasaki, H. (2001b) Bafilomycin A(1) inhibits rhinovirus infection in human airway epithelium: effects on endosome and ICAM-1. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **280**, L1115–L1127.

Takeda, M., Leser, G. P., Russell, C. J. & Lamb, R. A. (2003) Inaugural article: Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc. Natl Acad. Sci. USA*, **100**, 14610–14617.

Tarlo, S. M., Broder, I., Corey, P., Chan-Yeung, M., Ferguson, A., Becker, A., Rogers, C., Okada, M. & Manfreda, J. (2001) The role of symptomatic colds in asthma exacerbations: influence of outdoor allergens and air pollutants. *J. Allergy Clin. Immunol*., **108**, 52–58.

Thomas, K. W., Monick, M. M., Staber, J. M., Yarovinsky, T., Carter, A. B. & Hunninghake, G. W. (2002) Respiratory syncytial virus inhibits apoptosis and induces NF-kappa B activity through a phosphatidylinositol 3-kinase-dependent pathway. *J. Biol. Chem*., **277**, 492–501.

Tortorolo, L., Langer, A., Polidori, G., Vento, G., Stampachiacchere, B., Aloe, L. & Piedimonte, G. (2005) Neurotrophin overexpression in lower airways of infants with respiratory syncytial virus infection. *Am. J. Respir. Crit. Care Med*., **172**, 233–237.

Tripp, R. A. (2004) Pathogenesis of respiratory syncytial virus infection. *Viral Immunol*., **17**, 165–181.

Tripp, R. A., Moore, D., Winter, J. & Anderson, L. J. (2000) Respiratory syncytial virus infection and G and/or SH protein expression contribute to substance P, which mediates inflammation and enhanced pulmonary disease in BALB/c mice. *J. Virol*., **74**, 1614–1622.

Tripp, R. A., Jones, L. P., Haynes, L. M., Zheng, H., Murphy, P. M. & Anderson, L. J. (2001) CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. *Nat. Immunol*., **2**, 732–738.

Tristram, D. A., Hicks, W. & Hard, R. (1998) Respiratory syncytial virus and human bronchial epithelium. *Arch. Otolaryngol. Head Neck. Surg*., **124**, 777–83.

Van Riel, D., Munster, V. J., De Wit, E., Rimmelzwaan, G. F., Fouchier, R. A. M., Osterhaus, A. D. M. E. & Kuiken, T. (2006) H5N1 virus attachment to lower respiratory tract. *Science*, **312**, 399.

Wang, X., Li, M., Zheng, H., Muster, T., Palese, P., Beg, A. A. & Garcia-Sastre, A. (2000) Influenza A virus NS1 protein prevents activation of NF-kappa B and induction of alpha/beta interferon. *J. Virol*., **74**, 11566–11573.

Wark, P. A. B., Johnston, S. L., Bucchieri, F., Powell, R., Puddicombe, S., Laza-Stanca, V., Holgate, S. T. & Davies, D. E. (2005) Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J. Exp. Med*., **201**, 937–947.

Weinstock, J. V., Blum, A., Metwali, A., Elliott, D., Bunnett, N. & Arsenescu, R. (2003) Substance P regulates Th1-type colitis in IL-10 knockout mice. *J. Immunol*., **171**, 3762–3767.

Wilkinson, T. M. A., Hurst, J. R., Perera, W. R., Wilks, M., Donaldson, G. C. & Wedzicha, J. A. (2006) Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest*, **129**, 317–324.

Wohlleben, G., Muller, J., Tatsch, U., Hambrecht, C., Herz, U., Renz, H., Schmitt, E., Moll, H. & Erb, K. J. (2003) Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J. Immunol*., **170**, 4601–4611.

Wu, S. & Kaufman, R. J. (1996) Double-stranded (ds) RNA binding and not dimerization correlates with the activation of the dsRNA-dependent protein kinase (PKR). *J. Biol. Chem*., **271**, 1756–1763.

Wurzer, W. J., Planz, O., Ehrhardt, C., Giner, M., Silberzahn, T., Pleschka, S. & Ludwig, S. (2003) Caspase 3 activation is essential for efficient influenza virus propagation. *EMBO J*., **22**, 2717–2728.

Yang, D., Chertov, O., Bykovskaia, S. N., Chen, Q., Buffo, M. J., Shogan, J., Anderson, M., Schrouder, J. M., Wang, J. M., Howard, O. M. Z. & Oppenheim, J. J. (1999) β-defensins: linking innate and adaptive immunity through dendritic and t cell CCR6. *Science*, **286**, 525–528.

Zhang, L., Peeples, M. E., Boucher, R. C., Collins, P. L. & Pickles, R. J. (2002) Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J. Virol*., **76**, 5654–66.

Zhang, W., Yang, H., Kong, X., Mohapatra, S., San Juan-Vergara, H., Hellermann, G., Behera, S., Singam, R., Lockey, R. F. & Mohapatra, S. S. (2005) Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nat. Med*., **11**, 56–62.

Zhang, Y., Luxon, B. A., Casola, A., Garofalo, R. P., Jamaluddin, M. & Brasier, A. R. (2001) Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. *J. Virol*., **75**, 9044–9058.

Zheng, S., De, B. P., Choudhary, S., Comhair, S. A. A., Goggans, T., Slee, R., Williams, B. R. G., Pilewski, J., Haque, S. J. & Erzurum, S. C. (2003) Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis. *Immunity*, **18**, 619–630.

13

Bacterial Interactions with the Airway Epithelium

Marisa I. Gómez and **Alice Prince**
Department of Pediatrics, Columbia University, New York, USA

13.1 Introduction

The airway epithelium represents a primary site for the introduction and deposition of potentially pathogenic micro-organisms into the body, through inspired air. The epithelial mucosa is an important component of the innate immune system that recognizes conserved structures in microorganisms, called pathogen-associated molecular patterns (PAMPs), and initiates appropriate signaling to recruit and activate phagocytic cells to the airway. In spite of the innate defences of the respiratory tract, under certain circumstances, bacterial pathogens reach the lower airway and cause disease. Most people aspirate to some degree while sleeping and oropharyngeal secretions may enter the lower respiratory tract, but due to the numerous defence mechanisms that exist in the airway, especially mucociliary clearance, most aspirated material is of no clinical significance. However, alterations in mucosal barriers, such as impaired ciliary action, mechanical trauma and inflammatory changes induced by viral infection, predispose the lung to pneumonia. Impairment of the immune system, either in humoral or cell-mediated immunity, or phagocytic function, facilitates colonization at the lower respiratory tract.

13.2 Bacterial pneumonia

Community acquired pneumonia involves airway pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus*, and *Moraxella catharralis* (Andrews *et al*., 2003) and it is often due to aspiration of oropharyngeal secretions containing colonizing flora. Previous viral infections can induce changes in the respiratory epithelium that impair mucociliary clearance and can serve to increase susceptibility to subsequent bacterial infection (McCullers, 2006). Health care associated infections, such as nosocomial pneumonias

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

are an increasingly common entity often due to opportunistic pathogens such as *Pseudomonas aeruginosa* and Enterobacteria (Schwartz, 2004). Ventilator-associated pneumonia is a major problem in the intensive care unit setting and is commonly caused by methicillin-resistant *S. aureus* (MRSA), as well by as Gram-negative bacteria such as *P. aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii* (Shaw, 2005). These species form biofilms and adhere to plastic, which facilitates colonization and subsequent lung infection. Once the patient is intubated, the natural barrier between the oropharynx and trachea is bypassed and the epithelium is damaged as a result of the mechanical injury associated with endotracheal intubation. These conditions favour attachment and growth of bacteria and allow for greatly increased bacterial density. Intubation and sedation of the patient impairs normal cough-mediated clearance and facilitates the entry of colonizing pathogens through micro- and macro-aspiration of infected oral and gastric contents (Craven, 2000). Among Gram-positive bacteria, *S. aureus* is a major cause of pneumonia in hospitalized patients and is becoming increasingly resistant to antibiotics. Between 40 and 60 per cent of all hospital *S. aureus* isolates are resistant to methicillin and intermediate to high levels of resistance to vancomycin have also been recently described (Chang *et al*., 2003; Craven, 2000; Lindsay and Holden, 2004; Tenover, 2006).

Most of the pathology associated with chronic lung diseases such as COPD and cystic fibrosis (CF) are due to bacterial infection and inflammation. The course of COPD is characterized by intermittent exacerbations responsible for the morbidity and mortality associated with this disease (Murphy, 2006). It is estimated that half of the exacerbations are caused by bacterial infection, particularly non-encapsulated *H. influenzae, Moraxella catarrhalis*, and *S. pneumoniae* (Sethi and Murphy, 2001). In CF, dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel, in airway epithelium and submucosal glands leads to dehydrated secretions and predisposes to infection and chronic inflammation in the respiratory tract. This is manifested early in life by airway obstruction and recurrent infections of the lung and paranasal sinuses, often due to *S. aureus* and *H. influenzae* (Goss and Rosenfeld, 2004). The CF lung is particularly susceptible to *P. aeruginosa*, and this organism plays a critical role in the development and progression of pulmonary disease in these patients.

13.3 Bacterial virulence factors: role in lung colonization

Colonization of the airway by pathogenic bacteria is the first step in the development of pneumonia. Pulmonary pathogens share a variety of virulence factors that allow them to adhere to the airway epithelium and colonize the airway (Table 13.1). Bacterial adhesins play a key role in colonization because they allow the bacteria to attach to airway cells. *P. aeruginosa* pili mediate epithelial adherence and are important in the pathogenesis of airway infection particularly during invasive infection. Piliated *P. aeruginosa*, but not *pil* mutants, can colonize neonatal mice and cause pulmonary inflammatory responses (Tang *et al*., 1995). The secretion of many *P. aeruginosa* toxins which act within eukaryotic cells also requires pilin-mediated attachment (Feldman *et al*., 1998; Hauser *et al*., 1998). Streptococcal and staphylococcal surface adhesins can bind to host cell matrix components, and to cellular receptors upregulated during lung inflammation (Bogaert *et al*., 2004; Foster, 2005; Foster and McDevitt, 1994).

Table 13.1 Bacterial virulence factors involved in airway colonization

Virulence factor	Bacteria
Cell wall associated adhesins	*Streptococcus pneumoniae*
	Staphylococcus aureus
	S. pneumoniae
Pili	*Pseudomonas aeruginosa*
Flagella	*P. aeruginosa*
Biofilm	*P. aeruginosa*
	S. aureus
Siderofores	*P. aeruginosa*
	S. aureus
Neuraminidase	*P. aeruginosa*
	S. pneumoniae
Hyaluronidase	*S. pneumoniae*

Pseudomonas strains that initially colonize the airway express flagella which provide motility and are highly immunostimulatory. Flagella are essential to establish infection and Fla^- mutants are less virulent in a mouse model of pneumonia, failing to disseminate throughout the lung or into the bloodstream (Feldman *et al*., 1998; Tseng *et al*., 2006). Flagella also bind to mucin through the cap protein FliD (Arora *et al*., 1998) and activate pro-inflammatory responses in immune cells of myeloid origin as well as epithelial cells (Wyant *et al*., 1999).

For successful colonization, bacteria need to acquire iron from host tissues, where it is tightly bound to transferrin or, in the airway, lactoferrin (Xiao and Kisaalita, 1997). Pathogens have developed a complex regulatory system to compete with lactoferrin for iron. *P. aeruginosa* siderophores, pyochelin and pyoverdin (Vasil and Ochsner, 1999; Xiao and Kisaalita, 1997) and the SirABC transporter in *S. aureus* (Dale *et al*., 2004) are examples of these iron-uptake systems.

The pulmonary pathogens *P. aeruginosa* and *S. aureus* live in free planktonic form, or in biofilms (Parsek and Singh, 2003; Yarwood and Schlievert, 2003), which are highly structured communities that coat surfaces, such as plastic catheters as well as the mucosal surface of the airway. The coordinated expression of diverse groups of genes within this community of bacteria is directed by small, diffusible molecules called quorum sensors. At low density, bacteria live in the planktonic form, but if the number of organism increases greatly, quorum sensors are secreted. Once a critical density of these molecules is achieved, they diffuse back into the organisms where, along with transcriptional activators, they direct the expression of virulence genes that allow the bacteria to evade the host response and survive as a community. The ability of *P. aeruginosa* to adapt to a biofilm mode of growth is determined by the availability of free iron (Banin *et al*., 2005), and the iron chelator lactoferrin inhibits highly structured biofilm formation (Singh *et al*., 2002), forcing bacteria to scavenge iron using siderophores. *P. aeruginosa* possess two major mechanisms for iron acquisition, the high-affinity pyoverdin system and the lower-affinity pyochelin system. Iron uptake by pyoverdine allows for a critical level of intracellular iron that serves as the signal for biofilm development, a mechanism that is mediated by the ferric uptake regulator Fur (Banin *et al*., 2005). Biofilm production by *S. aureus*, however, is induced in iron-restricted conditions, a mechanism also regulated by Fur (Johnson *et al*., 2005).

Biofilm production has an important role in bacterial persistence in the lung, especially in diseases like CF. In this and other chronic infections, bacterial adaptation to the environment results in the selection of organisms that are more persistent and less invasive, and the biofilm mode of growth plays a key role in this adaptation (Costerton *et al*., 1999; Hoiby *et al*., 2001; Singh *et al*., 2002; Singh *et al*., 2000). In the CF airway, mucin, cellular components from neutrophils, such as DNA and actin, as well as damaged airway epithelium serve as a biological matrix that facilitates growth of *P. aeruginosa* in biofilm (Landry *et al*., 2006; Walker *et al*., 2005). Mucin is one of the most abundant polymers in CF airway and *P. aeruginosa* has mucin-specific adhesins that mediate bacterium–mucin interactions. The flagellar cap protein FliD appears to have a prominent role in this interaction (Landry *et al*., 2006). Dehydrated mucus present in CF generates a unique environment in which bacteria are confined spatially. This increases the local concentration of autoinducers, leading to increased biofilm formation (Matsui *et al*., 2006).

Respiratory pathogens such as *S. pneumoniae* and *P. aeruginosa* produce neuraminidases, enzymes that cleaves terminal sialic acid from cell surface glycoconjugates (Vimr *et al*., 2004). While the role of viral neuraminidases in pathogenesis is well established (Colman, 1994), less is known about the contribution of bacterial neuraminidases to the development of pneumonia. Many pulmonary pathogens bind to asialylated glycolipids (Krivan *et al*., 1988), suggesting that the ability to desialylate mucosal surfaces could contribute to bacterial colonization of the airway. In fact, neuraminidase activity increases *S. pneumoniae* adherence and invasion by exposing receptors for the bacteria (McCullers and Bartmess, 2003; Tong *et al*., 2001). *P. aeruginosa*, a major pathogen in CF, is predominantly entrapped in airway secretions and bacterial attachment is not necessarily required to initiate inflammatory responses. The *P. aeruginosa* neuraminidase, instead of exposing host receptors, has a critical role in the initial colonization of the lung by targeting bacterial glycoconjugates and contributing to the formation of biofilm (Soong *et al*., 2006).

After initial colonization by bacteria, the airway epithelium senses the presence of bacterial products and initiates the inflammatory response that leads to the recruitment and activation of phagocytic cells into the airway. While PMNs function to eradicate infection, they also impede air exchange. Thus, the balance between efficient phagocytosis of inspired bacteria and airway compromise is physiologically critical. Lung injury associated with bacterial infection is usually the result of both the direct destructive effects of the organism on the lung parenchyma and damage due to host inflammatory responses.

13.4 Bacterial recognition by airway epithelial cells

Intact bacteria are rarely in direct contact with airway epithelial cells which are well protected by mucins. Following epithelial damage, bacteria may gain access to the epithelial surface or, as occurs in COPD and CF, elicit inflammation without invading the epithelium through shed bacterial components that stimulate surface-exposed or intracellular receptors (Figure 13.1; Table 13.2). Unlike other mucosal surfaces, the lower airway are normally sterile, and exposure to bacterial components triggers an inflammatory response. Airway epithelial cells are polarized, form tight junctions and have a compartmentalized distribution of surface receptors. Bacterial receptors must be exposed apically to recognize pathogens or bacterial products present in the lumen of the airway. These receptors are present in low density compared to those on immune cells, which is likely to prevent excessive responses that

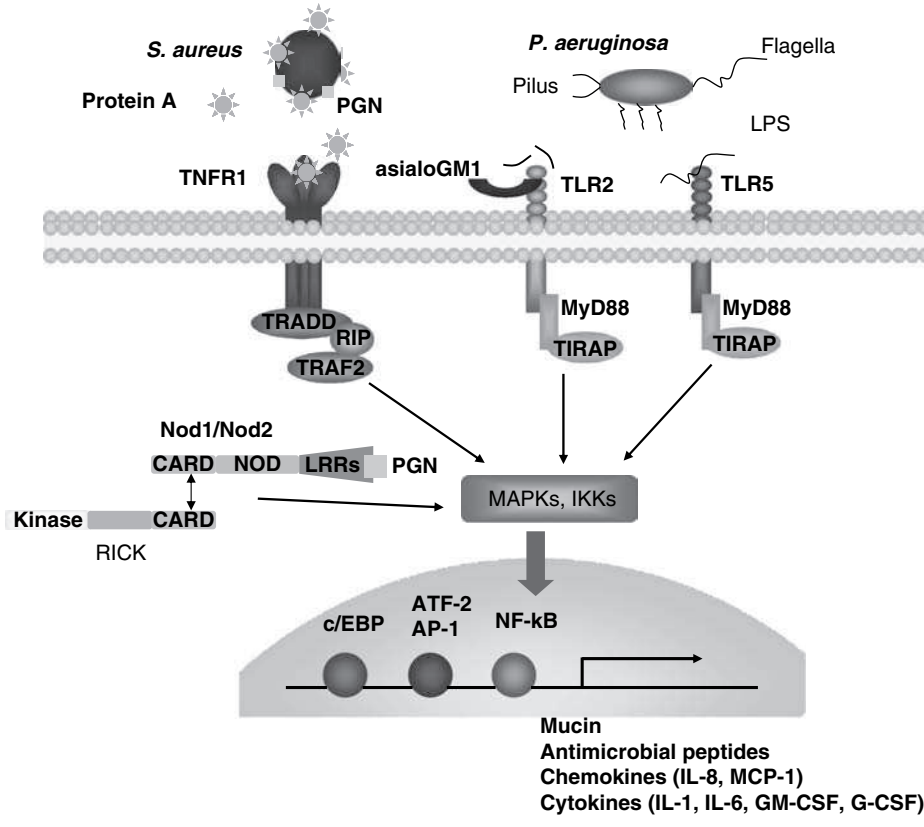


Figure 13.1 Airway epithelial responses to bacterial ligands. Bacterial ligands (Flagella, pilus, protein A, peptidoglycan (PGN)) are recognized by surface (asialoGM1, TLRs, TNFR1) or intracellular (Nods) receptors. Signaling cascades are initiated through adaptor proteins (MyD88/TIRAP, TRAM/TRIF, TRADD/RIP, RICK), and MAPK and IKK-dependent translocation of transcription factors leads to transcription of inflammatory mediators

Table 13.2 Immunostimulatory bacterial ligands and its receptors

Bacterial ligand	Receptor
Flagella, pili, Gram-positive and Gram-negative bacteria	AsialoGM1
Lipoteicoic acid, lipoproteins, flagella, lipoarabinomannan, phenol soluble modulins	TLR2
LPS, pneumolysin	TLR4
Flagella	TLR5, Ipaf
Protein A	TNFR1, EGFR
Peptidoglycan	NOD1/2

impede normal lung function. However, upon repeated bacterial stimulation more receptors are recruited to the apical surface where they initiate the inflammatory response when this is required to clear the infection.

13.4.1 Toll-like receptors

Among the eleven Toll-like receptors (TLRs) that have been recognized to date (Akira and Takeda, 2004; Takeda and Akira, 2004), TLRs 1–10 are expressed in airway epithelial cells (Greene *et al*., 2005; Muir *et al*., 2004). However, not all of these TLRs and the associated adaptors are equally available on the surface of the airway epithelium where they can respond to luminal contaminants (Chapter 9). TLR4 for example is intracellular, and MD2 expression is limited (Guillot *et al*., 2004), which explains the minimal response of epithelial cells to LPS. TLR2 is apically expressed in airway epithelial cells and TLR5 is rapidly mobilized in response to bacteria.

TLR2 in airway infection

TLR2 recognizes a variety of microbial components, including lipoproteins/lipopeptides, lipoteicoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria and a phenol-soluble modulin from *Staphylococci* (Akira and Takeda, 2004; Takeda and Akira, 2004). In the airway. TLR2 forms a receptor complex with the asialoganglioside gangliotetraosylceramide (Galb1, 2GalNacb1, 4Galb1, 4Gal1Cer) (asialoGM1) on the apical surface of epithelial cells within the context of lipid rafts (Soong *et al*., 2004). This glycolipid has an exposed GalNacb1-4Gal moiety that serves as a receptor for bacterial pili (DiMango *et al*., 1998), flagella (Feldman *et al*., 1998) and a large number of pulmonary pathogens, including *S. pneumoniae, S. aureus*, and *P. aeruginosa* (Krivan *et al*., 1988). TLR2 is present on the apical surface of polarized cells with tight junctions, and is mobilized into specialized lipid raft microdomains containing caveolin-1 after bacterial stimulation. The role of TLR2 in initiating pro-inflammatory signaling in professional immune cells, as well as in airway epithelial in vitro has been established (Greene *et al*., 2005; Muir *et al*., 2004; Soong *et al*., 2004; Takeda and Akira, 2004). In airway epithelial cells, TLR2 is also involved in early responses to *P. aeruginosa* flagella (Adamo *et al*., 2004). TLR2 and asialoGM1 initiate signaling in response to *S. aureus* and *P. aeruginosa* leading to the activation of NF-κB and IL-8 production in a MyD88-dependent manner. The lipid raft microdomain seems to be essential for signaling as suggested by the effects of filipin in inhibiting activation of IL-8 expression in response to bacteria (Soong *et al*., 2004).

TLR2 mRNA expression is upregulated in the lungs during both Gram-positive and Gram-negative infection (Kajikawa *et al*., 2005; Knapp *et al*., 2004; Power *et al*., 2004). In response to *S. aureus* systemic infection, TLR2 null mice have increased mortality (Takeuchi *et al*., 2000). However, the situation in the lung seems to be different. The response of TLR2 null mice to intranasally inoculated *S. pneumoniae* did not significantly differ from wild type mice (Knapp *et al*., 2004). Cytokine and chemokine production, and the overall inflammatory response, was modestly reduced in TLR2 null mice, but there was no difference in bacterial clearance. Similar results were obtained in a model of postinfluenza pneumococcal pneumonia (Dessing *et al*., 2006). A recent study using aerosolized *S. aureus* demonstrated the involvement of TLR2 in the production of inflammatory cytokines (TNF-α, IL-1β) and chemokines (KC, MIP-2), as well as in PMN recruitment. However, *S. aureus* clearance was not affected in MyD88 null mice (Skerrett *et al*., 2004b). TLR2 null mice did not show increased susceptibility to the Gram-negative pulmonary pathogen *P. aeruginosa* (Skerrett *et al*., 2007), indicating that other signaling pathways

in addition to TLR2/MyD88 signaling are evidently involved in responses to pulmonary pathogens.

Intracellular pathogens such as *Legionella pneumophila* are also handled by TLR2 (Akamine *et al*., 2005). The protective role of TLR2-mediated signaling in the clearance of *L. pneumophila* in the lungs was demonstrated using a murine model of Legionnaires' disease (Archer and Roy, 2006). In vivo growth of wild type *L. pneumophila* was enhanced in the lungs of TLR2-deficient mice, resulting in a delay in bacterial clearance whereas *L. pneumophila dot*A mutants that cannot replicate intracellularly were efficiently cleared (Archer and Roy, 2006).

TLR4 in airway infection

TLR4 is an essential receptor for LPS recognition by professional immune cells (Hoshino *et al*., 1999; Poltorak *et al*., 1998). Although TLR4 is abundant in airway epithelial cells, it is less critical in signaling epithelial responses to *P. aeruginosa* (Muir *et al*., 2004; Soong *et al*., 2004). Airway epithelial cells, like other mucosal epithelia, are not particularly responsive to LPS as compared to myeloid cells, even when all of the required co-receptors and LPS binding proteins are provided (DiMango *et al*., 1995; Guillot *et al*., 2004). This limited responsiveness is probably due to the intracellular localization of TLR4 in airway epithelial cells, and low levels of expression of MD2 (Guillot *et al*., 2004). The lack of TLR4 involvement in epithelial responses to LPS in vitro does not imply that the lung itself is unresponsive. Nuclear translocation of NF-κB in response to inhaled LPS was observed in the bronchiolar epithelium of wild type mice. This response was not observed in transgenic mice expressing a dominant negative IKBβ in the airway epithelium (Skerrett *et al*., 2004a). Thus, the pulmonary response to LPS provided systemically (rather than by inhalation) can be mediated by TLR4 expressed by pulmonary endothelial cells (Andonegui *et al*., 2003).

TLR4 expression is increased during Gram-negative infection (Kajikawa *et al*., 2005; Knapp *et al*., 2004; Power *et al*., 2004) and this TLR plays an important role in the overall defences against *P. aeruginosa*. TLR4/MyD88 signaling is critical for the induction of inflammatory cytokines (TNF-α, IL-1β) and chemokines (KC, MIP-2), PMN recruitment to the lungs, and bacterial clearance in a mouse model of pneumonia (Power *et al*., 2004; Skerrett *et al*., 2004b). While early responses to *P. aeruginosa* were TLR4/MyD88-dependent (Power *et al*., 2004), a later response mediated by either TLR2 or MyD88-independent pathways has been proposed (Power *et al*., 2006). TLR4 contributes to a protective innate immune response to *H. influenzae* (Wang *et al*., 2002) and *K. pneumoniae* (Branger *et al*., 2004; Schurr *et al*., 2005). In addition to recognition of Gram-negative pathogens, TLR4 can play a modest role in the protective immune responses to pneumococcal pneumonia (Branger *et al*., 2004) and is apparently involved in pneumolysin signaling (Malley *et al*., 2003). However, the protective role of TLR4 during bacterial pneumonia is likely due to signaling in professional immune cells or endothelial cells rather than epithelial cells in the lungs.

TLR5 in airway infection

TLR5 recognizes flagellin, the principal component of flagella, from both Gram-positive and Gram-negative bacteria (Hayashi *et al*., 2001; Smith *et al*., 2003; Zhang *et al*., 2005). TLR5, although expressed in airway epithelial cells, is not abundant on the apical surface

but can be recruited following exposure to flagella, which can recognize asialoGM1/TLR2 as well (Adamo *et al*., 2004). TLR5 may be more abundant in basolateral compartments in some epithelial cells (Gewirtz, 2006; Gewirtz *et al*., 2001) where it only responds to invasive pathogens. The importance of TLR5 in mediating pro-inflammatory responses to flagella and clearance of *P. aeruginosa* during lung infection has been recently demonstrated in vivo using TLR5 null mice (Feuillet *et al*., 2006). Wild type mice showed neutrophil infiltration and a significant increase in pro-inflammatory cytokines and chemokines in response to intranasal inoculation with flagella, whereas neutrofil infiltration was absent in TLR5 null mice (Feuillet *et al*., 2006).

Multiple signaling pathways involving different TLRs are triggered in response to bacterial stimulation (Figure 13.1). Individual TLRs can activate distinct signaling cascades depending on the adaptor proteins involved. Although most TLR pro-inflammatory signaling in the lungs is MyD88-dependent, MyD88-independent signaling is also involved in NF-κB activation (Jiang *et al*., 2005; Kawai *et al*., 1999; Kawai and Akira, 2006; Yamamoto *et al*., 2004). MyD88 null mice have a more severe phenotype than null mice for any of the individual TLRs (Feng *et al*., 2003; Skerrett *et al*., 2007). This suggests that multiple TLRs contribute to the host response to certain organisms or other receptors not described yet can signal through MyD88 and participate in bacterial responses.

13.4.2 TNF-α receptor 1

Bacteria also exploit endogenous signaling cascades to initiate airway inflammation. Staphylococci recognizes TNF-α receptor 1 (TNFR1) and activate TNF signaling in airway cells (Gómez *et al*., 2004). Staphylococcal protein A signaling through TNFR1 plays a central role in the pathogenesis of *S. aureus* pneumonia. Staphylococcal mutants lacking protein A do not cause pneumonia in a mouse model of infection, nor are the mice lacking TNFR1 susceptible to staphylococcal pneumonia. The absence of TNFR1-dependent PMN recruitment prevents morbidity due to the pathological consequences of excessive PMN accumulation into the airway. The requirement for protein A-TNFR1 signaling in the development of pneumonia is consistent with the observations that TLR2 and MyD88 signaling is not essential in protection from staphylococcal infection (Knapp *et al*., 2004; Skerrett *et al*., 2004b).

13.4.3 Intracellular receptors

In addition to cell surface receptors that recognize microbial components, mammalian cells also have NOD-like receptors (NLRs) to recognize PAMPs in the cytosol of infected cells. NLRs include proteins such as NOD1 (nucleotide-binding oligomerization domain 1), NOD2, NALPs (NACHT-, LRR-and pyrin-domain-containing proteins) and IPAF (ICE-protease activating factor) (Inohara *et al*., 2004; Kufer *et al*., 2006; Kufer and Sansonetti, 2007; Mariathasan and Monack, 2007). Nod1 and Nod2 are both expressed by airway epithelial cells. NOD1 recognizes peptidoglycans containing meso-diaminopimelate acid (DAP) found mainly in Gram-negative bacteria (Chamaillard *et al*., 2003; Girardin *et al*., 2003a), whereas NOD2 mediates responsiveness to muramyldipeptide MurNac-L-Ala-D-iso-Gln (MDP) conserved in peptidoglycans of all bacteria (Girardin *et al*., 2003b; Inohara *et al*., 2003). *S. pneumoniae* invades epithelial and endothelial cells activating the Nod signaling and accordingly lung expression of Nod2 is upregulated during *S. pneumoniae* infection in mice (Opitz

et al., 2004; Schmeck *et al*., 2004). In vitro experiments demonstrate that NF-κB activation by *S. pneumoniae* is dependent on NOD2 and mediated by IRAK and IRAK2. These results suggest that signaling through NODs is a major pathway through which *S. pneumoniae* induces inflammation in the lungs.

The intracellular pattern-recognition receptor Ipaf has been recently identified as essential sensor for cytoplasmic flagellin in macrophages (Amer *et al*., 2006; Franchi *et al*., 2006; Miao *et al*., 2006). Upon activation Ipaf form a multiprotein complex, an inflammasome that serves as a platform for the activation of caspase 1 which in turn process IL-β (Martinon and Tschopp, 2007; Ogura *et al*., 2006) a pro-inflammatory cytokine that it is rapidly secreted in response to bacterial infections. Activation of caspase-1 through Ipaf during *L. pneumophila* infection, restricts bacterial replication within the macrophages by regulating phagosome maturation (Amer *et al*., 2006; Franchi *et al*., 2006; Miao *et al*., 2006).

Caspase-1 and caspase-5 are also activated by NALPs (Sutterwala *et al*., 2006). The NALP3 inflammasome is activated in response to bacterial pore-forming toxins such as listeriolysin (Mariathasan *et al*., 2006) and aerolysin (Gurcel *et al*., 2006). This inflammasome senses the efflux of potassium as the result of the pore formation in the cell membrane and initiates activation of central regulators of membrane biogenesis (SREBPs) which promote cell survival upon toxin challenge (Gurcel *et al*., 2006). *S. aureus* activates the NALP3 inflammasome by a mechanism that seems to be independent of pore-forming toxin release (Mariathasan *et al*., 2006) suggesting that bacterial components might be internalized and recognized by NALP3. The role of these inflammasomes in mediating responses to bacterial components in airway epithelial cells remains to be established.

13.5 Airway epithelial cell responses to bacteria

Epithelial cells signal the presence of bacterial components and secrete pro-inflammatory cytokines and chemokines that recruit immune cells to the site of infection and activate them. Several bacterial components are highly immunostimulatory, such as flagella, lipoproteins and staphylococcal protein A (Table 13.2). In response to bacteria, airway epithelial cells secrete numerous pro-inflammatory chemokines and cytokines such as neutrophil chemokine IL-8, the cytokines IL-6 and IL-1β, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocye colony stimulating factor (G-CSF) and transforming growth factor α (TGF-α) and β (TGF-β).

IL-1 and TNF-α, which is mainly produced by immune cells in the lung, induce the activation of nuclear factor κB (NF-κB) which promotes the expression of IL-8 and IL-6 as well as mucin (Strieter *et al*., 2002). Pro-inflammatory signaling is required to recruit and activate phagocytic cells to the site of infection. However, the relative balance between stimulating sufficient phagocytic cells to clear infection and overwhelming the lung with inflammation is critical in the development of fatal pneumonia. Whereas TNF-α has a protective effect in animal models of *P. aeruginosa* infection (Gosselin *et al*., 1995), TNFR null mice have decreased inflammation and increased rates of *P. aeruginosa* clearance (Skerrett *et al*., 1999). Similarly, IL-1 receptor null mice intranasally inoculated with *P. aeruginosa* were found to have greater bacterial clearance in their lungs and reduced bacteremia, as compared to wild type mice (Schultz *et al*., 2002).

Neutrophil chemokines, in particular IL-8, play a critical role in recruitment and maintenance of leukocytes during infection. Animal models of pneumonia have demonstrated

an increase in chemokines in bronchoalveolar lavage of infected mice and blockade of chemokine receptors results in reduced neutrophil infiltration and clearance of bacteria in the lung, and increased mortality (Mehrad and Standiford, 1999; Strieter *et al*., 2002). Transgenic mice engineered for enhanced expression of KC (the mouse equivalent of IL-8) and Groα/MIP-2 have improved survival during bacterial pneumonia (Tsai *et al*., 1998).

As part of the inflammatory response, airway epithelial cells also express adhesion molecules, such as the intercellular adhesion molecule (ICAM)-1, to allow the adhesion of recruited neutrophils. Neutrophils are involved in the recognition, phagocytosis and clearance of bacteria. This is accomplished by opsonization through Fc-mediated binding, or antigen recognition using complement receptors. The pathogen is ingested and killed in the PMN phagosome through the expression of peptides and reactive oxygen intermediates. Thus, neutrophils are critically important to phagocytose and kill bacteria. However, their own lysis and release of elastase is a potent stimulus of epithelial IL-8 which promotes a cycle of continued inflammation (Nakamura *et al*., 1992).

The cytokines G-CSF and GM-CSF are also expressed by airway epithelial cells and are important in activating PMNs and dendritic cells that have been recruited at the site of infection and enhance their survival by inhibition of apoptosis (Saba *et al*., 2002). GM-CSF-deficient mice have significantly increased susceptibility to streptococcal infection (LeVine *et al*., 1999). Later during infection, and as a consequence of IL-6 trans-signaling, epithelial cells secrete the chemokine monocyte chemottractant protein-1 (MCP-1) which plays an important role in the resolution of inflammation and orchestrating the initiation of the adaptive immune response (Gómez *et al*., 2005; Hurst *et al*., 2001; Strieter *et al*., 2003).

Antimicrobial peptides are also induced by contact of airway epithelial cells with bacterial products or pro-inflammatory mediators. They have a broad spectrum of activity against Gram-positive and Gram-negative bacteria and show synergistic activity with other host defence molecules, such as lysosyme and lactoferrin (Bals and Hiemstra, 2004). It has been shown that expression of human beta defensin (hBD)-2, hBD-3, hBD4, the cathelicidin-derived peptide LL37, and several other antimicrobial peptides are induced in vivo during pneumonia (Bals *et al*., 2001; Hiratsuka *et al*., 1998). In vitro studies showed upregulation of hBD-2 in by primary airway epithelial cells in response to *P. aeruginosa* LPS and to inflammatory cytokines such as IL-1β and TNF-α (Becker *et al*., 2000; Harder *et al*., 2000; Singh *et al*., 1998). *P. aeruginosa* and TNF-α both induced expression of hBD-3 in lung cells in vitro (Harder *et al*., 2001) or cultured fetal lung explants (Jia *et al*., 2001). Analysis of the putative promoter regions of hBD-2 and hBD-3 genes reveals consensus sequence sites for the regulators of transcription NF-κB, NF-IL-6 and AP-1 (Becker *et al*., 2000; Diamond *et al*., 2000) which are activated in airway epithelial cells by bacterial ligands. In fact, TLR2 signaling has been linked to the induction of hBD-2 in tracheobronchial cells (Hertz *et al*., 2003) and primary airway epithelial cells (Wang *et al*., 2003).

Other inducible host defence molecules include mucins and reactive nitrogen species, such as nitric oxide (NO) (Rochelle *et al*., 1998). Bacterial components, such as flagellin, bind to mucin (Arora *et al*., 1998; Landry *et al*., 2006; Scharfman *et al*., 2001) and are cleared by the mucociliary escalator. Mucin concentration in broncoalveolar lavage (BAL) is increased in response to LPS and flagella, and MUC5A gene expression is upregulated by LPS and Gram-positive and Gram-negative bacteria (Dohrman *et al*., 1998), as well as by pro-inflammatory chemokines and cytokines expressed by airway epithelial cells (Voynow

et al., 2006). MUC2 expression is also increased in response to *P. aeruginosa* LPS and flagellin (Li *et al*., 1997; McNamara *et al*., 2001), *S. aureus* lipoteichoic acid (Lemjabbar and Basbaum, 2002), and *H. influenzae* (Jono *et al*., 2002), by signaling cascades that share common elements with those induced by TLRs.

NO is produced by inducible nitric oxide synthase (iNOS) expressed by neutrophils, macrophages and epithelial cells following stimulation with bacteria, LPS and cytokines (Warner *et al*., 1995). NO has important local effects in vasodilatation as well as direct anti-bacterial activities (Darling and Evans, 2003; Mehta, 2005). Decreased iNOS expression contributes to bacterial colonization and infection as demonstrated in vitro; CF airway epithelial cells, which have reduced expression of iNOS, were transfected with human iNOS cDNA and bacterial killing activity was restored (Darling and Evans, 2003). In addition, iNOS has anti-inflammatory activity by virtue of its effects in preventing neutrophil recruitment into the lungs (Hickey *et al*., 1997).

13.6 Signaling pathways involved in chemokine and cytokine production by epithelial cells

The signaling pathways activated through TLRs, TNFR1 and Nod proteins in airway epithelial cells resemble the cascades activated via these receptors in immune cells (Hehlgans and Pfeffer, 2005; Takeda and Akira, 2004) (Figure 13.1). TLR signaling is mediated by MyD88, IRAK and TRAF6, and all these molecules are recruited to the receptor complex with asialoGM1 in lipid rafts domains (Soong *et al*., 2004). Activation of this pathway leads to the nuclear translocation of NF-κB and transcription of pro-inflammatory genes. MyD88-independent pathways that involve TRAM and TRIF are also activated in the airway in response to TLR4 ligands (Fischer *et al*., 2006; Jiang *et al*., 2005). The MyD88-independent/TRIF-dependent cascade regulates production of IFN-β and IFN inducible genes (Toshchakov *et al*., 2002). Protein A signaling through TNFR1 resembles TNF signaling with recruitment of TRADD, RIP and TRAF2 to the receptor and activation of p38 and JNK MAPK and ATF-2 phosphorylation and translocation to the nucleus (Gómez *et al*., 2004). Less is known about signaling through intracellular receptors. Nod2-mediated responses to *S. pneumoniae* that induce NF-κB activation seem to be mediated by IRAK-TRAF5-NIK, TAB2 and TAK1 (Opitz *et al*., 2004; Schmeck *et al*., 2004).

The pulmonary pathogens *S. aureus* and *P. aeruginosa* activate Ca^{2+} fluxes in epithelial cells upon contact with specific receptors. TLR2 ligation on airway cells stimulates release of Ca^{2+} from intracellular stores by activating TLR2 phosphorylation by c-Src which leads to the recruitment of PI3K and PLC-γ and subsequent Ca^{2+} release (Chun and Prince, 2006). These Ca^{2+} fluxes are sufficient to activate NF-κB and generate IL-8 and GM-CSF expression (Ratner *et al*., 2001; Saba *et al*., 2002). Several other Ca^{2+}-dependent transcription factors are also activated by bacterial ligands leading to local cytokine expression and mucin production (McNamara *et al*., 2001). Peptidoglycan activates the leucine zipper containing transcription factors cAMP-responsive element-binding protein (CREB)/ATF and AP-1 (Gupta *et al*., 1999). CREB senses changes in cyclic nucleotides released at the surface of the airway in response to Ca^{2+} fluxes. In addition, CREB functions as a co-activator of CCAAT/enhancer binding protein (C/EBP) which regulates the expression of IL-6 (Kovacs *et al*., 2003). Ca^{2+}-dependent signaling could provide a target for immunomodulatory therapy.

13.7 Regulation of inflammation by epithelial cells – receptor shedding

Airway epithelial cells also regulate pro-inflammatory signaling. The pulmonary pathogens *S. aureus* and *P. aeruginosa* induce activation of TACE, the TNF-α converting enzyme, in airway epithelial cells (Gómez *et al*., 2004, 2005). TACE or ADAM 17 is a member of the ADAM family of proteases involved in the release of several superficial proteins including the TNF-α, EGF and IL-6 receptors (Mezyk *et al*., 2003). TACE has an important role in regulation of inflammation. TACE cleaves TNFR1 from the surface of airway epithelial cells and macrophages (Gómez *et al*., 2004) and this shed, soluble TNFR1 serves to neutralize free TNF-α (mainly produced by immune cells) and protein A in the airway lumen, as well as to prevent further epithelial activation through loss of TNFR1 from the cell. Soluble TNFR1 also exerts immunoregulatory functions by induction of apoptosis in monocytes through reverse signaling via membrane-bound TNF-α (Waetzig *et al*., 2005).

Bacterial ligands regulate the activity of TACE itself. *S. aureus* protein A induces TACE activation by direct binding to the epidermal growth factor receptor (EGFR) (Gómez *et al*., 2007). EGFR signaling stimulates the ERK1/2 MAPK, which in turn phosphorylates TACE. While TACE is highly expressed on the apical surface of airway epithelial cells, the substrate, TNFR1, has to be mobilized to the surface where it co-localizes with TACE. Thus, staphylococcal recognition of EGFR and activation of TACE serves to counteract the pro-inflammatory consequences of TNR1 signaling, PMN recruitment and activation.

Bacterial activation of TACE also induces shedding of the IL-6Rα from epithelial cells and trans-signaling (Gómez *et al*., 2005). Epithelial responsiveness to IL-6 is dependent upon the presence of two receptors, gp130 and IL-6Rα (gp80) (Bauer *et al*., 1989; Heinrich *et al*., 2003). Shed soluble IL-6Rα binds to IL-6 forming a ligand–receptor complex that interacts with membrane-bound gp130 in a high-affinity interaction termed ‘trans-signaling’. This interaction initiates MCP-1 expression by epithelial cells, which heralds the shift from acute inflammation (PMN recruitment) to a resolution phase with macrophage/monocyte signaling and clearance of apoptotic PMNs (Amano *et al*., 2004; Hurst *et al*., 2001). In addition, shed IL-6R induces a decrease in IL-8 production (Hurst *et al*., 2001; Marin *et al*., 2001) probably due to STAT5-dependent inhibition of NF-κB (Luo and Yu-Lee, 2000).

As the control of inflammation in the airway is crucial, the activation of both pro- and anti-inflammatory responses by mucosal epithelial cells has a primary role in determining the outcome of pneumonia. Airway epithelial cells not only regulate their own signaling capabilities shed epithelial receptors can serve to decrease the pro-inflammatory signaling induced by immune cells in the lung.

13.8 Lung damage and bacterial invasion of the airway epithelium

Airway colonization can be followed by bacterial invasion, bacteremia and mortality in hosts with immunorepressed mucosal barrier function. *P. aeruginosa* produces a number of toxins that are delivered into the host cell cytosol by the type III secretion system. The type III secretion system is associated with acute invasive infection and requires pilin-mediated bacterial-epithelial cell contact (Hauser *et al*., 1998). This system consists of three

components: the secretion apparatus, the translocation or targeting apparatus, and the secreted toxins (effector proteins) and cognate chaperons (Gauthier *et al*., 2003). The four type III-secreted proteins that have been identified in *P. aeruginosa* are: exotoxins U, Y, S and T. Almost no strain encodes all four of them, but all strains express exotoxin T (ExoT) (Shaver and Hauser, 2004), suggesting a more conserved role for this protein in pathogenesis. Mutants lacking ExoT exhibit reduced virulence and do not disseminate (Garrity-Ryan *et al*., 2004; Hauser *et al*.,1998). ExoT, as well as ExoS, encodes an N-terminal GTPase-activating protein that targets the small GTPases Rho, Rac, and Cdc, and a C-terminal ADP ribosyltransferase (ADPRT) domain (Barbieri and Sun, 2004). ExoT and ExoS modulate many processes that involve the actin cytoskeleton and are key in maintaining the mucosal barrier integrity. ExoT also inhibits host-cell division by targeting cytokinesis (Shafikhani and Engel, 2006). ExoU has recently been characterized as a member of the phospholipase A family of enzymes with potent cytotoxin activity (McMorran *et al*., 2003; Sato and Frank, 2004).

13.9 Conclusions

Bacterial pathogens interact with airway epithelial cells by expressing numerous ligands called PAMPs that elicit inflammatory responses through surface-exposed and intracellular receptors. Failure of the normal innate clearance mechanisms enables organisms to persist in the airway lumen. Both adherent bacteria and shed products are potent stimuli for epithelial pro-inflammatory chemokine and cytokine production. This serves to recruit PMNs from the circulation into the airway. Recruitment of PMNs to the lung is critical to eradicate respiratory pathogens, but is not innocuous to the host. Inflammation is detrimental to the major function of the airway in maintaining an open conduit for gas exchange. Indeed, much more so than at other mucosal surfaces. Thus, the balance between efficient phagocytosis of inspired bacteria and airway compromise is physiologically critical and determines the outcome of lung infections.

References

Adamo, R., Sokol, S., Soong, G., Gómez, M.I. and Prince, A. (2004) *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *Am J Respir Cell Mol Biol*, **30**, 627–634.

Akamine, M., Higa, F., Arakaki, N., Kawakami, K., Takeda, K., Akira, S. and Saito, A. (2005) Differential roles of Toll-like receptors 2 and 4 in in vitro responses of macrophages to *Legionella pneumophila. Infect Immun*, **73**, 352–361.

Akira, S. and Takeda, K. (2004) Toll-like receptor signaling. *Nat Rev Immunol*, **4**, 499–511.

Amano, H., Morimoto, K., Senba, M., Wang, H., Ishida, Y., Kumatori, A., Yoshimine, H., Oishi, K., Mukaida, N. and Nagatake, T. (2004) Essential contribution of monocyte chemoattractant protein-1/C-C chemokine ligand-2 to resolution and repair processes in acute bacterial pneumonia. *J Immunol*, **172**, 398–409.

Amer, A., Franchi, L., Kanneganti, T.D., Body-Malapel, M., Ozoren, N., Brady, G., Meshinchi, S., Jagirdar, R., Gewirtz, A., Akira, S. and Nunez, G. (2006) Regulation of *Legionella* phagosome maturation and infection through flagellin and host Ipaf. *J Biol Chem*, **281**, 35217–35223.

Andonegui, G., Bonder, C.S., Green, F., Mullaly, S.C., Zbytnuik, L., Raharjo, E. and Kubes, P. (2003) Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest*, **111**, 1011–1020.

Andrews, J., Nadjm, B., Gant, V. and Shetty, N. (2003) Community-acquired pneumonia. *Curr Opin Pulm Med*, **9**, 175–180.

Archer, K.A. and Roy, C.R. (2006) MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of *Legionella pneumophila* in a mouse model of Legionnaires' disease. *Infect Immun*, **74**, 3325–3333.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S. and Ramphal, R. (1998) The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun*, **66**, 1000–1007.

Bals, R. and Hiemstra, P.S. (2004) Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J*, **23**, 327–333.

Bals, R., Weiner, D.J., Meegalla, R.L., Accurso, F. and Wilson, J.M. (2001) Salt-independent abnormality of antimicrobial activity in cystic fibrosis airway surface fluid. *Am J Respir Cell Mol Biol*, **25**, 21–25.

Banin, E., Vasil, M.L. and Greenberg, E.P. (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A*, **102**, 11076–11081.

Barbieri, J.T. and Sun, J. (2004) *Pseudomonas aeruginosa* ExoS and ExoT. *Rev Physiol Biochem Pharmacol*, **152**, 79–92.

Bauer, J., Bauer, T.M., Kalb, T., Taga, T., Lengyel, G., Hirano, T., Kishimoto, T., Acs, G., Mayer, L. and Gerok, W. (1989) Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the expression in human hepatocytes. *J Exp Med*, **170**, 1537–1549.

Becker, M.N., Diamond, G., Verghese, M.W. and Randell, S.H. (2000) CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem*, **275**, 29731–29736.

Bogaert, D., De Groot, R. and Hermans, P.W. (2004) *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis*, **4**, 144–154.

Branger, J., Knapp, S., Weijer, S., Leemans, J.C., Pater, J.M., Speelman, P., Florquin, S. and van der Poll, T. (2004) Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect Immun*, **72**, 788–794.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M.A., Foster, S.J., Mak, T.W., Nunez, G. and Inohara, N. (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol*, **4**, 702–707.

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R., Brown, W.J., Cardo, D. and Fridkin, S.K. (2003) Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *N Engl J Med*, **348**, 1342–1347.

Chun, J. and Prince, A. (2006) Activation of Ca^{2+}-dependent signaling by TLR2. *J Immunol*, **177**, 1330–1337.

Colman, P.M. (1994) Influenza virus neuraminidase: structure, antibodies, and inhibitors. *Protein Sci*, **3**, 1687–1696.

Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science*, **284**, 1318–1322.

Craven, D.E. (2000) Epidemiology of ventilator-associated pneumonia. *Chest*, **117**, 186S–187S.

Dale, S.E., Sebulsky, M.T. and Heinrichs, D.E. (2004) Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus. J Bacteriol*, **186**, 8356–8362.

Darling, K.E. and Evans, T.J. (2003) Effects of nitric oxide on *Pseudomonas aeruginosa* infection of epithelial cells from a human respiratory cell line derived from a patient with cystic fibrosis. *Infect Immun*, **71**, 2341–2349.

Dessing, M.C., van der Sluijs, K.F., Florquin, S., Akira, S. and van der Poll, T. (2006) Toll-like Receptor2 does not contribute to host response during postinfluenza pneumococcal pneumonia. *Am J Respir Cell Mol Biol*., **36**, 609–614.

Diamond, G., Kaiser, V., Rhodes, J., Russell, J.P. and Bevins, C.L. (2000) Transcriptional regulation of beta-defensin gene expression in tracheal epithelial cells. *Infect Immun*, **68**, 113–119.

DiMango, E., Zar, H.J., Bryan, R. and Prince, A. (1995) Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J Clin Invest*, **96**, 2204–2210.

DiMango, E., Ratner, A.J., Bryan, R., Tabibi, S. and Prince, A. (1998) Activation of NF-kappaB by adherent *Pseudomonas aeruginosa* in normal and cystic fibrosis respiratory epithelial cells. *J Clin Invest*, **101**, 2598–2605.

Dohrman, A., Miyata, S., Gallup, M., Li, J.D., Chapelin, C., Coste, A., Escudier, E., Nadel, J. and Basbaum, C. (1998) Mucin gene (MUC 2 and MUC 5AC) upregulation by Gram-positive and Gram-negative bacteria. *Biochim Biophys Acta*, **1406**, 251–259.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H. and Prince, A. (1998) Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun*, **66**, 43–51.

Feng, C.G., Scanga, C.A., Collazo-Custodio, C.M., Cheever, A.W., Hieny, S., Caspar, P. and Sher, A. (2003) Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. *J Immunol*, **171**, 4758–4764.

Feuillet, V., Medjane, S., Mondor, I., Demaria, O., Pagni, P.P., Galan, J.E., Flavell, R.A. and Alexopoulou, L. (2006) Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proc Natl Acad Sci U S A*, **103**, 12487–12492.

Fischer, H., Yamamoto, M., Akira, S., Beutler, B. and Svanborg, C. (2006) Mechanism of pathogen-specific TLR4 activation in the mucosa: fimbriae, recognition receptors and adaptor protein selection. *Eur J Immunol*, **36**, 267–277.

Foster, T.J. (2005) Immune evasion by staphylococci. *Nat Rev Microbiol*, **3**, 948–958.

Foster, T.J. and McDevitt, D. (1994) Surface-associated proteins of *Staphylococcus aureus*: their possible roles in virulence. *FEMS Microbiol Lett*, **118**, 199–205.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.D., Ozoren, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E.P. and Nunez, G. (2006) Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol*, **7**, 576–582.

Garrity-Ryan, L., Shafikhani, S., Balachandran, P., Nguyen, L., Oza, J., Jakobsen, T., Sargent, J., Fang, X., Cordwell, S., Matthay, M.A. and Engel, J.N. (2004) The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. *Infect Immun*, **72**, 546–558.

Gauthier, A., Thomas, N.A. and Finlay, B.B. (2003) Bacterial injection machines. *J Biol Chem*, **278**, 25273–25276.

Gewirtz, A.T. (2006) Immune responses to flagellin in Crohn's disease: good, bad, or irrelevant? *Am J Physiol Gastrointest Liver Physiol*. **292**, G706–G710.

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J. and Madara, J.L. (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol*, **167**, 1882–1885.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., Coyle, A.J., DiStefano, P.S., Bertin, J., Sansonetti, P.J. and Philpott, D.J. (2003a) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science*, **300**, 1584–1587.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J. and Sansonetti, P.J. (2003b) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem*, **278**, 8869–8872.

Gómez, M.I., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A. and Prince, A. (2004) *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat Med*, **10**, 842–848.

Gómez, M.I., Sokol, S.H., Muir, A.B., Soong, G., Bastien, J. and Prince, A.S. (2005) Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling. *J Immunol*, **175**, 1930–1936.

Gómez, M.I., Seaghdha, M.O. and Prince, A.S. (2007) *Staphylococcus aureus* protein A activates TACE through EGFR-dependent signaling. *Embo J*. **26**, 701–709.

Goss, C.H. and Rosenfeld, M. (2004) Update on cystic fibrosis epidemiology. *Curr Opin Pulm Med*, **10**, 510–514.

Gosselin, D., DeSanctis, J., Boule, M., Skamene, E., Matouk, C. and Radzioch, D. (1995) Role of tumor necrosis factor alpha in innate resistance to mouse pulmonary infection with *Pseudomonas aeruginosa*. *Infect Immun*, **63**, 3272–3278.

Greene, C.M., Carroll, T.P., Smith, S.G., Taggart, C.C., Devaney, J., Griffin, S., O'Neill S, J. and McElvaney, N.G. (2005) TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol*, **174**, 1638–1646.

Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M. and Si-Tahar, M. (2004) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem*, **279**, 2712–2718.

Gupta, D., Wang, Q., Vinson, C. and Dziarski, R. (1999) Bacterial peptidoglycan induces CD14-dependent activation of transcription factors CREB/ATF and AP-1. *J Biol Chem*, **274**, 14012–14020.

Gurcel, L., Abrami, L., Girardin, S., Tschopp, J. and van der Goot, F.G. (2006) Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. *Cell*, **126**, 1135–1145.

Harder, J., Meyer-Hoffert, U., Teran, L.M., Schwichtenberg, L., Bartels, J., Maune, S. and Schroder, J.M. (2000) Mucoid *Pseudomonas aeruginosa*, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol*, **22**, 714–721.

Harder, J., Bartels, J., Christophers, E. and Schroder, J.M. (2001) Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem*, **276**, 5707–5713.

Hauser, A.R., Fleiszig, S., Kang, P.J., Mostov, K. and Engel, J.N. (1998) Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. *Infect Immun*, **66**, 1413–1420.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M. and Aderem, A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, **410**, 1099–1103.

Hehlgans, T. and Pfeffer, K. (2005) The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology*, **115**, 1–20.

Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G. and Schaper, F. (2003) Principles of interleukin (IL)-6-type cytokine signaling and its regulation. *Biochem J*, **374**, 1–20.

Hertz, C.J., Wu, Q., Porter, E.M., Zhang, Y.J., Weismuller, K.H., Godowski, P.J., Ganz, T., Randell, S.H. and Modlin, R.L. (2003) Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol*, **171**, 6820–6826.

Hickey, M.J., Sharkey, K.A., Sihota, E.G., Reinhardt, P.H., Macmicking, J.D., Nathan, C. and Kubes, P. (1997) Inducible nitric oxide synthase-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. *FASEB J*, **11**, 955–964.

Hiratsuka, T., Nakazato, M., Date, Y., Ashitani, J., Minematsu, T., Chino, N. and Matsukura, S. (1998) Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. *Biochem Biophys Res Commun*, **249**, 943–947.

Hoiby, N., Krogh Johansen, H., Moser, C., Song, Z., Ciofu, O. and Kharazmi, A. (2001) *Pseudomonas aeruginosa* and the in vitro and in vivo biofilm mode of growth. *Microbes Infect*, **3**, 23–35.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*, **162**, 3749–3752.

Hurst, S.M., Wilkinson, T.S., McLoughlin, R.M., Jones, S., Horiuchi, S., Yamamoto, N., Rose-John, S., Fuller, G.M., Topley, N. and Jones, S.A. (2001) IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity*, **14**, 705–714.

Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S.J., Moran, A.P., Fernandez-Luna, J.L. and Nunez, G. (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem*, **278**, 5509–5512.

Inohara, N., Chamaillard, M., McDonald, C. and Nunez, G. (2004) NOD-LRR proteins: role in host–microbial interactions and inflammatory disease. *Annu Rev Biochem*. **74**, 355–383.

Jia, H.P., Schutte, B.C., Schudy, A., Linzmeier, R., Guthmiller, J.M., Johnson, G.K., Tack, B.F., Mitros, J.P., Rosenthal, A., Ganz, T. and McCray, P.B., Jr. (2001) Discovery of new human beta-defensins using a genomics-based approach. *Gene*, **263**, 211–218.

Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M. and Beutler, B. (2005) CD14 is required for MyD88-independent LPS signaling. *Nat Immunol*, **6**, 565–570.

Johnson, M., Cockayne, A., Williams, P.H. and Morrissey, J.A. (2005) Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves fur-dependent and fur-independent mechanisms. *J Bacteriol*, **187**, 8211–8215.

Jono, H., Shuto, T., Xu, H., Kai, H., Lim, D.J., Gum, J.R., Jr., Kim, Y.S., Yamaoka, S., Feng, X.H. and Li, J.D. (2002) Transforming growth factor-beta -Smad signaling pathway cooperates with NF-kappa B to mediate nontypeable *Haemophilus influenzae*-induced MUC2 mucin transcription. *J Biol Chem*, **277**, 45547–45557.

Kajikawa, O., Frevert, C.W., Lin, S.M., Goodman, R.B., Mongovin, S.M., Wong, V., Ballman, K., Daubeuf, B., Elson, G. and Martin, T.R. (2005) Gene expression of Toll-like receptor-2, Toll-like receptor-4, and MD2 is differentially regulated in rabbits with *Escherichia coli* pneumonia. *Gene*, **344**, 193–202.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K. and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*, **11**, 115–122.

Kawai, T. and Akira, S. (2006) TLR signaling. *Cell Death Differ*, **13**, 816–825.

Knapp, S., Wieland, C.W., van't Veer, C., Takeuchi, O., Akira, S., Florquin, S. and van der Poll, T. (2004) Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J Immunol*, **172**, 3132–3138.

Kovacs, K.A., Steinmann, M., Magistretti, P.J., Halfon, O. and Cardinaux, J.R. (2003) CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation. *J Biol Chem*, **278**, 36959–36965.

Krivan, H.C., Roberts, D.D. and Ginsburg, V. (1988) Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. *Proc Natl Acad Sci U S A*, **85**, 6157–6161.

Kufer, T.A. and Sansonetti, P.J. (2007) Sensing of bacteria: NOD a lonely job. *Curr Opin Microbiol*, **10**, 92–69.

Kufer, T.A., Banks, D.J. and Philpott, D.J. (2006) Innate immune sensing of microbes by Nod proteins. *Ann N Y Acad Sci*, **1072**, 19–27.

Landry, R.M., An, D., Hupp, J.T., Singh, P.K. and Parsek, M.R. (2006) Mucin–*Pseudomonas aeruginosa* interactions promote biofilm formation and antibiotic resistance. *Mol Microbiol*, **59**, 142–151.

Lemjabbar, H. and Basbaum, C. (2002) Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* in epithelial cells. *Nat Med*, **8**, 41–46.

LeVine, A.M., Reed, J.A., Kurak, K.E., Cianciolo, E. and Whitsett, J.A. (1999) GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection. *J Clin Invest*, **103**, 563–569.

Li, J.D., Dohrman, A.F., Gallup, M., Miyata, S., Gum, J.R., Kim, Y.S., Nadel, J.A., Prince, A. and Basbaum, C.B. (1997) Transcriptional activation of mucin by *Pseudomonas aeruginosa* lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. *Proc Natl Acad Sci U S A*, **94**, 967–972.

Lindsay, J.A. and Holden, M.T. (2004) *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol*, **12**, 378–385.

Luo, G. and Yu-Lee, L. (2000) Stat5b inhibits NFkappaB-mediated signaling. *Mol Endocrinol*, **14**, 114–123.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R. and Golenbock, D.T. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A*, **100**, 1966–1971.

Mariathasan, S. and Monack, D.M. (2007) Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat Rev Immunol*, **7**, 31–40.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M. and Dixit, V.M. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*, **440**, 228–232.

Marin, V., Montero-Julian, F.A., Gres, S., Boulay, V., Bongrand, P., Farnarier, C. and Kaplanski, G. (2001) The IL-6-soluble IL-6Ralpha autocrine loop of endothelial activation as an intermediate between acute and chronic inflammation: an experimental model involving thrombin. *J Immunol*, **167**, 3435–3442.

Martinon, F. and Tschopp, J. (2007) Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ*, **14**, 10–22.

Matsui, H., Wagner, V.E., Hill, D.B., Schwab, U.E., Rogers, T.D., Button, B., Taylor, R.M., 2nd, Superfine, R., Rubinstein, M., Iglewski, B.H. and Boucher, R.C. (2006) A physical linkage between cystic fibrosis airway surface dehydration and *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A*, **103**, 18131–18136.

McCullers, J.A. (2006) Insights into the interaction between influenza virus and pneumococcus. *Clin Microbiol Rev*, **19**, 571–582.

McCullers, J.A. and Bartmess, K.C. (2003) Role of neuraminidase in lethal synergism between influenza virus and *Streptococcus pneumoniae*. *J Infect Dis*, **187**, 1000–1009.

McMorran, B., Town, L., Costelloe, E., Palmer, J., Engel, J., Hume, D. and Wainwright, B. (2003) Effector ExoU from the type III secretion system is an important modulator of gene expression in lung epithelial cells in response to *Pseudomonas aeruginosa* infection. *Infect Immun*, **71**, 6035–6044.

McNamara, N., Khong, A., McKemy, D., Caterina, M., Boyer, J., Julius, D. and Basbaum, C. (2001) ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proc Natl Acad Sci U S A*, **98**, 9086–9091.

Mehrad, B. and Standiford, T.J. (1999) Role of cytokines in pulmonary antimicrobial host defense. *Immunol Res*, **20**, 15–27.

Mehta, S. (2005) The effects of nitric oxide in acute lung injury. *Vascul Pharmacol*, **43**, 390–403.

Mezyk, R., Bzowska, M. and Bereta, J. (2003) Structure and functions of tumor necrosis factor-alpha converting enzyme. *Acta Biochim Pol*, **50**, 625–645.

Miao, E.A., Alpuche-Aranda, C.M., Dors, M., Clark, A.E., Bader, M.W., Miller, S.I. and Aderem, A. (2006) Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol*, **7**, 569–575.

Muir, A., Soong, G., Sokol, S., Reddy, B., Gomez, M.I., Van Heeckeren, A. and Prince, A. (2004) Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol*, **30**, 777–783.

Murphy, T.F. (2006) The role of bacteria in airway inflammation in exacerbations of chronic obstructive pulmonary disease. *Curr Opin Infect Dis*, **19**, 225–230.

Nakamura, H., Yoshimura, K., McElvaney, N.G. and Crystal, R.G. (1992) Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest*, **89**, 1478–1484.

Ogura, Y., Sutterwala, F.S. and Flavell, R.A. (2006) The inflammasome: first line of the immune response to cell stress. *Cell*, **126**, 659–662.

Opitz, B., Puschel, A., Schmeck, B., Hocke, A.C., Rosseau, S., Hammerschmidt, S., Schumann, R.R., Suttorp, N. and Hippenstiel, S. (2004) Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae. J Biol Chem*, **279**, 36426–36432.

Parsek, M.R. and Singh, P.K. (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol*, **57**, 677–701.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, **282**, 2085–2088.

Power, M.R., Peng, Y., Maydanski, E., Marshall, J.S. and Lin, T.J. (2004) The development of early host response to *Pseudomonas aeruginosa* lung infection is critically dependent on myeloid differentiation factor 88 in mice. *J Biol Chem*, **279**, 49315–49322.

Power, M.R., Marshall, J.S., Yamamoto, M., Akira, S. and Lin, T.J. (2006) The myeloid differentiation factor 88 is dispensable for the development of a delayed host response to *Pseudomonas aeruginosa* lung infection in mice. *Clin Exp Immunol*, **146**, 323–329.

Ratner, A.J., Bryan, R., Weber, A., Nguyen, S., Barnes, D., Pitt, A., Gelber, S., Cheung, A. and Prince, A. (2001) Cystic fibrosis pathogens activate Ca^{2+}-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J Biol Chem*, **276**, 19267–19275.

Rochelle, L.G., Fischer, B.M. and Adler, K.B. (1998) Concurrent production of reactive oxygen and nitrogen species by airway epithelial cells in vitro. *Free Radic Biol Med*, **24**, 863–868.

Saba, S., Soong, G., Greenberg, S. and Prince, A. (2002) Bacterial stimulation of epithelial G-CSF and GM-CSF expression promotes PMN survival in CF airways. *Am J Respir Cell Mol Biol*, **27**, 561–567.

Sato, H. and Frank, D.W. (2004) ExoU is a potent intracellular phospholipase. *Mol Microbiol*, **53**, 1279–1290.

Scharfman, A., Arora, S.K., Delmotte, P., Van Brussel, E., Mazurier, J., Ramphal, R. and Roussel, P. (2001) Recognition of Lewis x derivatives present on mucins by flagellar components of *Pseudomonas aeruginosa. Infect Immun*, **69**, 5243–5248.

Schmeck, B., Zahlten, J., Moog, K., van Laak, V., Huber, S., Hocke, A.C., Opitz, B., Hoffmann, E., Kracht, M., Zerrahn, J., Hammerschmidt, S., Rosseau, S., Suttorp, N. and Hippenstiel, S. (2004) *Streptococcus pneumoniae*-induced p38 MAPK-dependent phosphorylation of RelA at the interleukin-8 promotor. *J Biol Chem*, **279**, 53241–53247.

Schultz, M.J., Rijneveld, A.W., Florquin, S., Edwards, C.K., Dinarello, C.A. and van der Poll, T. (2002) Role of interleukin-1 in the pulmonary immune response during *Pseudomonas aeruginosa* pneumonia. *Am J Physiol Lung Cell Mol Physiol*, **282**, L285–290.

Schurr, J.R., Young, E., Byrne, P., Steele, C., Shellito, J.E. and Kolls, J.K. (2005) Central role of toll-like receptor 4 signaling and host defense in experimental pneumonia caused by Gram-negative bacteria. *Infect Immun*, **73**, 532–545.

Schwartz, D.B. (2004) Hospital-acquired pneumonia-evolving knowledge. *Curr Opin Pulm Med*, **10** (Suppl 1), S9–13.

Sethi, S. and Murphy, T.F. (2001) Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. *Clin Microbiol Rev*, **14**, 336–363.

Shafikhani, S.H. and Engel, J. (2006) *Pseudomonas aeruginosa* type III-secreted toxin ExoT inhibits host-cell division by targeting cytokinesis at multiple steps. *Proc Natl Acad Sci U S A*, **103**, 15605–15610.

Shaver, C.M. and Hauser, A.R. (2004) Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. *Infect Immun*, **72**, 6969–6977.

Shaw, M.J. (2005) Ventilator-associated pneumonia. *Curr Opin Pulm Med*, **11**, 236–241.

Singh, P.K., Jia, H.P., Wiles, K., Hesselberth, J., Liu, L., Conway, B.A., Greenberg, E.P., Valore, E.V., Welsh, M.J., Ganz, T., Tack, B.F. and McCray, P.B., Jr. (1998) Production of beta-defensins by human airway epithelia. *Proc Natl Acad Sci U S A*, **95**, 14961–14966.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature*, **407**, 762–764.

Singh, P.K., Parsek, M.R., Greenberg, E.P. and Welsh, M.J. (2002) A component of innate immunity prevents bacterial biofilm development. *Nature*, **417**, 552–555.

Skerrett, S.J., Martin, T.R., Chi, E.Y., Peschon, J.J., Mohler, K.M. and Wilson, C.B. (1999) Role of the type 1 TNF receptor in lung inflammation after inhalation of endotoxin or *Pseudomonas aeruginosa. Am J Physiol*, **276**, L715–727.

Skerrett, S.J., Liggitt, H.D., Hajjar, A.M., Ernst, R.K., Miller, S.I. and Wilson, C.B. (2004a) Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. *Am J Physiol Lung Cell Mol Physiol*, **287**, L143–152.

Skerrett, S.J., Liggitt, H.D., Hajjar, A.M. and Wilson, C.B. (2004b) Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against *Pseudomonas aeruginosa* but not *Staphylococcus aureus. J Immunol*, **172**, 3377–3381.

Skerrett, S.J., Wilson, C.B., Liggitt, H.D. and Hajjar, A.M. (2007) Redundant Toll-like receptor signaling in the pulmonary host response to *Pseudomonas aeruginosa. Am J Physiol Lung Cell Mol Physiol*, **292**, L312–322.

Smith, K.D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M.A., Barrett, S.L., Cookson, B.T. and Aderem, A. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat Immunol*, **4**, 1247–1253.

Soong, G., Reddy, B., Sokol, S., Adamo, R. and Prince, A. (2004) TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J Clin Invest*, **113**, 1482–1489.

Soong, G., Muir, A., Gomez, M.I., Waks, J., Reddy, B., Planet, P., Singh, P.K., Kaneko, Y., Wolfgang, M.C., Hsiao, Y.S., Tong, L. and Prince, A. (2006) Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. *J Clin Invest*, **116**, 2297–2305.

Strieter, R.M., Belperio, J.A. and Keane, M.P. (2002) Cytokines in innate host defense in the lung. *J Clin Invest*, **109**, 699–705.

Strieter, R.M., Belperio, J.A. and Keane, M.P. (2003) Host innate defenses in the lung: the role of cytokines. *Curr Opin Infect Dis*, **16**, 193–198.

Sutterwala, F.S., Ogura, Y., Szczepanik, M., Lara-Tejero, M., Lichtenberger, G.S., Grant, E.P., Bertin, J., Coyle, A.J., Galan, J.E., Askenase, P.W. and Flavell, R.A. (2006) Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity*, **24**, 317–327.

Takeda, K. and Akira, S. (2004) TLR signaling pathways. *Semin Immunol*, **16**, 3–9.

Takeuchi, O., Hoshino, K. and Akira, S. (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J Immunol*, **165**, 5392–5396.

Tang, H., Kays, M. and Prince, A. (1995) Role of *Pseudomonas aeruginosa* pili in acute pulmonary infection. *Infect Immun*, **63**, 1278–1285.

Tenover, F.C. (2006) Mechanisms of antimicrobial resistance in bacteria. *Am J Infect Control*, **34**, S3–10; discussion S64–73.

Tong, H.H., James, M., Grants, I., Liu, X., Shi, G. and DeMaria, T.F. (2001) Comparison of structural changes of cell surface carbohydrates in the eustachian tube epithelium of chinchillas infected with a *Streptococcus pneumoniae* neuraminidase-deficient mutant or its isogenic parent strain. *Microb Pathog*, **31**, 309–317.

Toshchakov, V., Jones, B.W., Perera, P.Y., Thomas, K., Cody, M.J., Zhang, S., Williams, B.R., Major, J., Hamilton, T.A., Fenton, M.J. and Vogel, S.N. (2002) TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol*, **3**, 392–398.

Tsai, W.C., Strieter, R.M., Wilkowski, J.M., Bucknell, K.A., Burdick, M.D., Lira, S.A. and Standiford, T.J. (1998) Lung-specific transgenic expression of KC enhances resistance to *Klebsiella pneumoniae* in mice. *J Immunol*, **161**, 2435–2440.

Tseng, J., Do, J., Widdicombe, J.H. and Machen, T.E. (2006) Innate immune responses of human tracheal epithelium to *Pseudomonas aeruginosa* flagellin, TNF-alpha, and IL-1beta. *Am J Physiol Cell Physiol*, **290**, C678–690.

Vasil, M.L. and Ochsner, U.A. (1999) The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol*, **34**, 399–413.

Vimr, E.R., Kalivoda, K.A., Deszo, E.L. and Steenbergen, S.M. (2004) Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev*, **68**, 132–153.

Voynow, J.A., Gendler, S.J. and Rose, M.C. (2006) Regulation of mucin genes in chronic inflammatory airway diseases. *Am J Respir Cell Mol Biol*, **34**, 661–665.

Waetzig, G.H., Rosenstiel, P., Arlt, A., Till, A., Brautigam, K., Schafer, H., Rose-John, S., Seegert, D. and Schreiber, S. (2005) Soluble tumor necrosis factor (TNF) receptor-1 induces apoptosis via reverse TNF signaling and autocrine transforming growth factor-beta1. *FASEB J*, **19**, 91–93.

Walker, T.S., Tomlin, K.L., Worthen, G.S., Poch, K.R., Lieber, J.G., Saavedra, M.T., Fessler, M.B., Malcolm, K.C., Vasil, M.L. and Nick, J.A. (2005) Enhanced *Pseudomonas aeruginosa* biofilm development mediated by human neutrophils. *Infect Immun*, **73**, 3693–3701.

Wang, X., Moser, C., Louboutin, J.P., Lysenko, E.S., Weiner, D.J., Weiser, J.N. and Wilson, J.M. (2002) Toll-like receptor 4 mediates innate immune responses to *Haemophilus influenzae* infection in mouse lung. *J Immunol*, **168**, 810–815.

Wang, X., Zhang, Z., Louboutin, J.P., Moser, C., Weiner, D.J. and Wilson, J.M. (2003) Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. *FASEB J*, **17**, 1727–1729.

Warner, R.L., Paine, R., 3rd, Christensen, P.J., Marletta, M.A., Richards, M.K., Wilcoxen, S.E. and Ward, P.A. (1995) Lung sources and cytokine requirements for in vivo expression of inducible nitric oxide synthase. *Am J Respir Cell Mol Biol*, **12**, 649–661.

Wyant, T.L., Tanner, M.K. and Sztein, M.B. (1999) *Salmonella typhi* flagella are potent inducers of proinflammatory cytokine secretion by human monocytes. *Infect Immun*, **67**, 3619–3624.

Xiao, R. and Kisaalita, W.S. (1997) Iron acquisition from transferrin and lactoferrin by *Pseudomonas aeruginosa* pyoverdin. *Microbiology*, **143**, 2509–2515.

Yamamoto, M., Takeda, K. and Akira, S. (2004) TIR domain-containing adaptors define the specificity of TLR signaling. *Mol Immunol*, **40**, 861–868.

Yarwood, J.M. and Schlievert, P.M. (2003) Quorum sensing in Staphylococcus infections. *J Clin Invest*, **112**, 1620–1625.

Zhang, Z., Louboutin, J.P., Weiner, D.J., Goldberg, J.B. and Wilson, J.M. (2005) Human airway epithelial cells sense *Pseudomonas aeruginosa* infection via recognition of flagellin by Toll-like receptor 5. *Infect Immun*, **73**, 7151–7160.

14

Interactions of Pollutants with the Epithelium

Ernst Wm. Spannhake
Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

14.1 Introduction

The respiratory epithelial cell surface represents the primary reactive interface of the body with pollutants suspended in the air. Given its tremendously large surface area, it serves as a very large target for interaction with toxic gases and particles in the airborne environment. Because the great majority of this surface is found in the most peripheral aspects of the lung, in the respiratory bronchioles and alveoli, determination of the functional consequences of interactions of these agents with epithelial cells and the cooperative roles played by other cells within the epithelial structures remains a challenge to researchers.

Due to their commonality of source, airborne pollutants are most often found in complex mixture with other chemicals and particles. This fact poses challenges to investigation of causality and mechanism of action in studies involving ambient, as opposed to controlled, experimental exposures. Conversely, it imposes a range of caveats that must be considered when the results of mechanistic, single pollutant studies carried out using *in vivo* or isolated cell systems are extrapolated to ambient exposures in humans.

The airborne pollutants focused on in this chapter, ozone, nitrogen dioxide, particulate matter, and secondhand cigarette smoke, are components of outdoor and indoor air that are of special current interest based on their involvement in the development and exacerbation of respiratory and cardiovascular disease in exposed populations. Although not the only pollutants in these environments, exposure to these agents are believed to pose particular risk to the health of individuals in both urban and rural communities within developed and developing countries.

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

14.2 Oxidant pollutants

The primary oxidant pollutant gases found in ambient outdoor air are ozone and nitrogen dioxide, the latter also being an important contaminant of indoor air. For the most part, these two gases share common modes of interaction with the airway epithelium, with their principal differences relating to the differing oxidizing capacity of each.

14.2.1 Cell surface activity

Ozone is the most highly reactive oxidant gas commonly found in the ambient environment. It is considered to be a secondary environmental pollutant, because it is typically formed by chemical reaction when primary combustion pollutants, such as nitrogen dioxide, react with volatile organic compounds in the presence of ultraviolet light. Ozone is a near-ubiquitous contaminant of outdoor air in areas of high vehicular and industrial emissions and, thus, represents a common component of airborne chemical exposure in both highly-developed and developing countries.

Most studies in human subjects support the concept that the deposition pattern of ozone tends to be greatest in the more peripheral, rather than central, airway, although deposition certainly occurs throughout the respiratory tract (Overton and Miller, 1988). Studies utilizing measures of pulmonary function thought to predominantly reflect changes in small airway, such as the $FEF_{25–75}$ and $V_{max\ 50}$ and $V_{max\ 75}$, tend to show measurable, and sometimes persistent, effects in peripheral regions, often at relatively low levels of exposure (Frank *et al*., 2001; McDonnell *et al*., 1991). The effectiveness of ozone in targeting the epithelium in the small airway may be enhanced by the decreased thickness and differing composition of epithelial lining fluid (ELF) in these regions.

The mechanisms through which ozone interacts with biological fluids and cell membranes have been the object of much investigation in the past 50 years. Studies suggested that the high reactivity of ozone with the variety of oxidizable molecular targets in ELF, including lipid, protein and carbohydrate moieties, makes it likely that much of the ozone that enters the respiratory tract will not interact directly with the surface of underlying epithelial cells (Menzel, 1976; Pryor and Church, 1991; Uppu and Pryor, 1994; Mudway and Kelly, 1998; Postlethwait *et al*., 1998; Ballinger *et al*., 2005; Kermani *et al*., 2006). Calculations (Pryor, 1992) further suggest that the majority of ozone reaching the epithelial cell membrane would be expected to react within this lipid-rich bilayer, with few ozone molecules entering the cell. The products from these reactions may themselves exhibit oxidant activity as reactive oxidant species (ROS), or may take on mediator roles at, or within, the epithelial cell membrane. For example, treatment of the BEAS-2B human bronchial epithelial cell line with selected products derived from the ozonation of phosphatidylcholine caused selective activation of cytosolic phospholipase (cPL)A2 and PLC and PLD (Kafoury *et al*., 1998). The ozone-induced activation of cPLA2 through this mechanism and its subsequent release of arachidonic acid from membrane phospholipids likely plays an important role in synthesis and release of prostaglandins, thromboxane B_2, leukotrienes C4, D_4, and E_4 (in some species), 12-HHT, and other eicosanoid products from human airway epithelial cells (McKinnon *et al*., 1993; Leikauf *et al*., 1993). Release of products of PLC activation, such as platelet activating factor, has also been reported (Kafoury *et al*., 1999). In addition, ozonolysis of lipids and other molecules within the ELF and epithelial cell membrane can lead to synthesis of reactive and bioactive products such as aldehydes, hydroxyhydroperoxides, and hydrogen

peroxide (Leikauf, 1995), cholesterol-derived oxysterols (Pulfer *et al*., 2005) and alkenals, especially 4-hydroxynonenal (Iles *et al*., 2005). An additional mechanism of ozone toxicity at the cell surface is its ability to oxidize sulfhydryl groups. In addition to targeting glutathione in extracellular regions, ozone can inhibit the function of enzymes, co-enzymes and other proteins by inactivating SH groups at their active sites (Mustafa, 1990).

Studies in human subjects of the effects of ozone at high ambient levels on mucociliary function do not indicate a measurable effect on overall particle clearance rates in the lung (Foster *et al*., 1987; Gerrity *et al*., 1993), suggesting that ozone surface chemistry may not affect this process to a great extent. However, it was noted that mucus flow in the more peripheral airway, which is thought to represent the predominant site of ozone deposition, demonstrated a significant increase following ozone (Foster *et al*., 1987). The basis for this peripheral lung effect could not be determined. In a study in which subjects were exposed to low and high ambient levels of ozone for 1 h, neither lung clearance rates nor measures of epithelial permeability were observed to be affected (Morrison *et al*., 2006).

Nitrogen dioxide exists as a free radical gas in the environment. It is produced by high-temperature combustion of nitrogen-containing fuels and is present in industrial, vehicular and home heating and cooking emissions. Its deposition in the respiratory tract is similar to that of ozone, with its broad distribution being primarily toward the peripheral airway. Reactive nitrogen species (RNS) closely linked to nitrogen dioxide in biological systems include nitric oxide (NO) and peroxynitrite ($ONOO^-$), the chemistry of which was recently reviewed by Pryor *et al*. (2006). With a redox potential half that of ozone, exogenous nitrogen dioxide targets molecules more easily oxidized, including unsaturated lipids and lipoproteins. Concentrations of the water-soluble antioxidants uric acid and ascorbic acid in ELF retrieved by lavage from healthy subjects were found to be significantly reduced following exposure of the fluid to nitrogen dioxide. This consumption was dose-dependent in the range of 0.05 to 1.0 ppm, whereas the decrease in glutathione levels was 10-fold less (Kelly and Tetley, 1997), in contrast to the high reactivity of glutathione with ozone (Kermani *et al*., 2006). Similarly, the decrease in both glutathione peroxidase (GPX) activity and protein levels in human ELF induced by ozone is not characteristic of nitrogen dioxide exposure (Avissar *et al*., 2000). These and similar studies underscore the extent to which the availability of susceptible targets in ELF plays in modulating the effects of nitrogen dioxide at the epithelial cell surface.

At high exposure levels, nitrogen dioxide has been shown to damage cilia and cause hypertrophy of the bronchial epithelium in animal inhalation experiments (Rombout *et al*., 1986). Although nitrogen dioxide reacts selectively and at moderate rates with non-radical targets, it reacts to a much greater extent with other radicals. Such activity allows for the development of cooperative oxidant interactions between RNS and ROS. Because nitrogen dioxide and ozone often exist as co-pollutants, these types of interactions may play an important role in epithelial cell activation and toxicity in the airway during ambient exposures (Pryor *et al*., 2006).

14.2.2 Stimulus transduction and intracellular activation

Although exposure to oxidant pollutants has been demonstrated in some studies to increase permeability in the airway of human subjects and in animal models (e.g. Broeckaert *et al*., 1999; van Bree *et al*., 2002; Voter *et al*., 2001) the mechanisms for this effect remain controversial. In *in vivo* systems, the relatively rapid influx of inflammatory cells makes

it difficult to separate direct effects on the airway epithelium from those resulting from subsequent inflammatory processes. Studies utilizing airway epithelial cells grown in culture at an air–liquid interface have provided the opportunity to investigate several key epithelial cell-specific responses in the absence of the confounding effects of other resident and transient cells (Yu *et al*., 1994). It is interesting to note that an increasing number of studies in this system suggest that airway epithelial cells from asthmatic donors respond differently than those from non-asthmatics. In one such study, permeability based on the movement of ^{14}C-BSA across the confluent cultures was seen to increase in cells from asthmatic, but not from non-asthmatic subjects in response to both ozone and nitrogen dioxide exposures (Bayram *et al*., 2002). These data suggest that inherent differences in susceptibility to oxidant challenge exist in asthmatic epithelial cells, and that these differences are preserved in epithelial cell culture in the absence of other cells. The basis for this apparent difference in responsiveness is not known; however, it has also been reported in studies of the release of granulocyte-monocyte colony-stimulating factor (GM-CSF), regulated upon activation, normal T-cell expressed and secreted (RANTES), and soluble intracellular adhesion molecule (ICAM)-1 from cultured bronchial epithelial cells derived from atopic asthmatic versus non-atopic non-asthmatic subjects (Bayram *et al*., 2001).

An early study made the surprising observation that pretreatment of normal human bronchial epithelial (HBE) cells with substance P (SP), a tachykinin released from nerves closely associated with the airway epithelium and implicated in the pathophysiology of asthma, inhibited the ozone-induced increase in transepithelial potential difference and inhibited paracellular mannitol flux. SP had no effect on either of these measures at baseline. The epithelial barrier protective effect of SP was mitigated by pretreatment with a neurokinin (NK)-1 receptor antagonist (Yu *et al*., 1996). That *in vitro* finding takes on added interest in light of a more recent study in which treatment of allergic asthmatics with an NK1/NK2 receptor antagonist enhanced, rather than mitigated, allergen-induced early and late airway responses (Boot *et al.,* 2007), suggesting that the neurokinin may play a protective role in those airway.

Given the wide range of reactive products generated by ozone and nitrogen dioxide, it is likely that the cellular signalling pathways that initiate their responses in the airway epithelial cell are shared to a large extent. The oxidant-related transcription factors NF-kappaB, NF-IL-6, and activator protein (AP)-1 appear to be involved in transduction of most of these signals. Exposure of cells of the A549 Type II-like epithelial line to ozone-induced DNA binding activity of all of these factors, followed by increases in interleukin (IL)-8 mRNA and protein levels. The activities of the transcription factors and IL-8 expression in response to ozone were demonstrated to be protein tyrosine kinase (PTK)- and protein kinase A (PKA)-dependent, but were independent of protein kinase C activity. These studies confirmed that the activity of the kinases was dependent upon reactive oxygen intermediates and that their activation was independent of one another (Jaspers *et al*., 1998). In cultures of human nasal epithelial cells exposed to a low to high ambient range of ozone, NF-kappaB showed a minimal dose–response activation that corresponded with ozone-induced free radical production. At high exposure levels, expression of tumour necrosis factor alpha (TNFα) was significantly increased in the cells (Nichols *et al*., 2001). In total, results of studies of oxidant exposures are consistent with the concept that the signalling pathways within epithelial cells have the potential for a high degree of regulatory interaction and that the level of oxidant stress applied may be a critical factor in determining the predominant pathway followed.

14.2.3 Stimulation of intercellular signalling

The ability of HBE cells to synthesize and release products from both apical and basolateral surfaces is extensive and well-documented in the literature. Under baseline conditions, these molecules modulate the activation and function of resident and transient cells in the airway mucosa. Under conditions of challenge from environmental pollutant agents, the patterns and magnitudes of expression of these mediators and cell-surface proteins can be dramatically altered, affecting innate host defence and susceptibility, immune and non-immune inflammatory responses, and the functional capacities of cells involved with structural changes in the lung. In addition to studies which employ exposure of cultured human cells, *in vivo* exposures followed by evaluation of biopsied respiratory mucosa provide similar expression data. For example, in nasal biopsies taken from healthy subjects 6 hours after exposure to a relatively high concentration of ozone (0.4 ppm, 2 h), levels of TNFα, IL-1β, IL-8, IL-6, granulocyte monocyte-colony stimulating factor (GM-CSF) and intercellular adhesion molecule (ICAM)-1 were increased in epithelial cells, as assessed by immunohistochemistry (Dokic and Howarth, 2006). Exposure to ambient levels of ozone has also been demonstrated to induce mediator release. Healthy subjects exposed to concentrations as low as 0.08 ppm for 6.6 h, with exercise, showed increased levels of IL-6 and PGE_2, as well as lactate dehydrogenase and alpha-1 antitrypsin in BAL fluid. PMN influx was also observed (Devlin *et al*., 1991).

Although somewhat controversial, synthesis and release of IL-5 has been reported from primary cultures of human bronchial and nasal epithelial cells (Salvi *et al*., 1999). This multi-functional cytokine is typically associated with immune activity involving basophils and eosinophils. In a cross-over study of healthy subjects exposed to a relatively low level of ozone (0.12 ppm, 2 h) or air, message for IL-5, whose product was localized in biopsies to the bronchial epithelial cells, was decreased by ozone. The upregulation of Th2 cytokines by nitrogen dioxide exposure has also been observed. In a study of repeated exposures of healthy non-smokers to 2 ppm for 4 h on four successive days, immunohistologic staining of biopsy tissue showed increased expression of IL-5, IL-10 and IL-13 in the bronchial epithelium (Pathmanathan *et al*., 2003). These effects on epithelial cell Th2 cytokine expression may represent one of the ways in which exposure to oxidant pollutants can modify airway allergic processes and may play a role in disease development in individuals, such as children indoors, who experience repeated exposure to low levels of nitrogen dioxide during early development. The presence of mediators in airway tissues may also predispose cells to injury. Inflammatory cytokines, whether released by the epithelial cells themselves or by other cells within the mucosa, have been shown to increase the toxicity of oxidant exposures. Pretreatment with TNFα and IFNγ resulted in a twofold to threefold increase in cytotoxicity of otherwise minimally-toxic levels of nitrogen dioxide in HBE cells (Ayyagari *et al*., 2004).

14.2.4 Associations with respiratory disease

A considerable body of data indicates that exposure of the respiratory system to ozone or nitrogen dioxide is associated with the development or exacerbation of lung-associated disease. In some cases, the role of the epithelium in mediating these effects is understood, while in other cases, the underlying mechanisms are not yet clear. Because of the relatively high reactivity of the oxidant pollutants with targets at the epithelial cell surface, it is

nevertheless likely that these cells are involved in transducing the signals that lead to these effects in most, if not all, situations of exposure.

The results of time-series studies of the relationship between ozone exposure in the ambient environment and cardiovascular and respiratory mortality have provided equivocal results. Two recent meta-analyses of these data support the notion that short-term exposure to ozone, independent of the levels of particulate matter present, is associated with mortality, particularly in the elderly (Bell *et al*., 2005; Ito *et al*., 2005). As air quality continues to decline in many urban areas linked to vehicular gas emissions, this association, if present, would take on increasing public health significance.

Many studies involving exposures of subjects to ozone under controlled conditions have demonstrated acute decrements in pulmonary function, even at commonly-found ambient levels (e.g. McDonnell *et al*., 1991). Furthermore, these and other functional studies confirm the small, peripheral airway as preferential targets. The observation that the effects of ozone in these regions may also persist beyond the exposure period and be cumulative with repeated exposure (Frank *et al*., 2001; Tager *et al*., 2005), place the epithelial lining in these areas at the nexus of intercellular signalling during exposures. Communication between epithelial cells and fibroblasts, and perhaps other cells associated with the epithelial-mesenchymal tropic unit, can be affected by exposure to ozone (Lang *et al*., 1998). Interference with normal growth processes in the developing lungs of infant monkeys undergoing chronic periodic exposure to ozone has been reported and suggests that exposure to ozone may alter the structure of the resulting lung, perhaps due to the superimposition of ozone-induced damage and repair cycles on the normal growth processes (Fanucchi *et al*., 2006; Schelegle *et al*., 2003).

One of the primary characteristics of allergic asthma is the presence of chronic airway inflammation. Because of the potent pro-inflammatory activity of ozone, it has been postulated that the superimposition of ozone exposure on asthmatic airway could lead to direct enhancement of the inflammatory state and provide a basis for disease exacerbation. Furthermore, the potential for ozone to co-operatively stimulate oxidant-related pathways already activated in epithelial, and other, cells of the asthmatic airway could provide a mechanism by which to impart to these cells an increased responsiveness to ozone exposure. Studies have employed both *in vivo* exposures of atopic asthmatic and normal subjects to ozone followed by bronchial biopsy and *in vitro* exposure of bronchial epithelial cells derived from similar cohorts. Among the proinflammatory mediators for which expression and release in response to ozone have been found to be significantly higher in an asthmatic as compared to control subjects are GM-CSF, IL-5, IL-8, epithelial neutrophil-activating protein (ENA)-78, and soluble ICAM (Bayram *et al*., 2001; Bosson *et al*., 2003).

There is considerable interest in the effect that exposure of the airway epithelium to ozone may have on responses to allergen challenge in allergic individuals. In one study, previously-sensitized and non-sensitized infant monkeys were exposed to 0.5 ppm ozone or filtered air for 8 hours per day for five consecutive days with or without concurrent allergen exposure on three of the five days. This protocol, with intervening 9-day washouts, was repeated 11 times over a 6-month period. The sensitized animals exposed to ozone showed marked increases in serum IgE and histamine levels and airway eosinophilia in response to allergen challenge compared to air-exposed counterparts. At the end of the 6-month study, ultrastructural changes in the airway of the ozone-exposed group were also apparent (Schelegle *et al*., 2003). In contrast, studies utilizing controlled exposures of human subjects to address this question have not provided definitive results. In one study, subjects with allergic rhinitis

and subjects with allergic asthma were exposed to ozone at near ambient or high ambient levels. High exposure protocols (single 3-h 0.25 ppm or 4-day repeated 0.125 ppm), but not single 0.125 ppm, were effective in increasing functional and inflammatory responsiveness to allergen challenge post exposure in both study groups (Holz *et al*., 2002). Using exposure protocols that result in a lower ozone dose, however, make alterations in allergen responsiveness in asthmatics more difficult to detect. Modification of exposure methodology in order to reduce the variability of the ozone concentration around the 1-h 0.12 ppm target concentration resulted in the loss of a previously-observed enhancement in the response to subsequent allergen challenge in six of nine allergic asthmatics (Hanania *et al*., 1998). In another study of 1-h exposures to 0.2 ppm, no effect on the early bronchoconstrictor response or on the late inflammatory response to allergen was observed in a group of 14 asthmatic subjects. However, it was noted that a subset of subjects whose initial pulmonary function decrement in response to ozone was the greatest tended to be most responsive to allergen following ozone (Chen *et al*., 2004). This observation has been reported by others, and suggests that individual variation in the ability of epithelial targets to mitigate ozone-induced oxidant stress may influence sensitivity to allergen challenge. It also appears that the concentration administered to the airway surface is an important determinant in initiating a detectable effect in susceptible individuals. Although most attention has focused on outdoor sources of ozone, it should be noted that some commercial 'air cleaner' products designed for indoor use generate ozone as part of their cleaning strategy. Some such devices release significant levels of ozone and may pose health risks for children with respiratory disease (Phillips *et al*., 1999).

In infants and young children in whom epithelial defence mechanisms may not be fully developed, there is particular concern regarding the effects of exposure to the oxidant pollutants, including nitrogen dioxide. In one of several studies using ambient measures of pollutants, nitrogen dioxide levels showed the largest association with asthma admissions in children from 5 to 14 years of age (Barnett *et al*., 2005). Chronic exposure of infants and young children to nitrogen dioxide is a particular issue for those who spend long periods in homes and other indoor environments in which combustion of nitrogen-containing fuels is used for cooking and heating, and in which cigarette smoke is present. Non-asthmatic infants living in homes at the highest quartile for nitrogen dioxide concentration (17.4 ppb) had a higher frequency of days with wheeze, persistent cough and shortness of breath than infants in the lowest quartile (van Strien *et al*., 2004). Increased likelihood of wheeze, shortness of breath and chest tightness was also seen in children with pre-existing asthma who were living in homes with nitrogen dioxide levels that were elevated, but below the current US ambient air quality standard (Belanger *et al*., 2006).

It may be that, in addition to the oxidant-induced synthesis and release of bronchoconstrictive agents by ozone and nitrogen dioxide, these pollutants exacerbate allergic responses through secondary pathways. As described earlier, both of these oxidants induce the release of the Th2 cytokines, IL-5, IL-10 and IL-13, from airway epithelial cells (Pathmanathan *et al*., 2003; Salvi *et al*., 1999). Under conditions of repeated release of these mediators associated with recurring exposures, patterns of cellular activity and the state of immune inflammation in the airway mucosa might be modified. Evidence has also been presented that oxidant pollutants may contribute to preexisting respiratory diseases by interacting with respiratory virus infections. The important role that virus infections play in asthma exacerbations is well known and is discussed in depth in Chapter 12. In a study in which personal nitrogen dioxide exposures were monitored every week for up to 13 months, episodes

of virus-associated upper respiratory-tract infections were linked to the severity of lower respiratory tract symptom scores. These investigators observed that the severity of virus-associated asthma exacerbations were greatest when the precipitating infection was preceded within a week by exposure to elevated levels of nitrogen dioxide. These elevated levels, whose median was 21 $\mu g\ m^{-3}$ in the highest tertile, were well within the current air quality standard (Chauhan *et al*., 2003).

In addition to other stimuli, both oxidant pollutants and several respiratory virus infections have been shown to increase expression of ICAM-1 on the surface of epithelial cells of the human upper and lower respiratory tract (Sajjan *et al*, 2006; Spannhake *et al*., 2002). As this adhesion molecule provides the point of attachment of the major receptor group human rhinoviruses (HRV) to the epithelial cell surface, its upregulation by oxidant pollutants could represent a means by which to increase the risk and spread of infection and the subsequent increase in asthma disease severity. To investigate mechanisms of interaction not linked to ICAM-1 binding, human upper and lower respiratory tract epithelial cells were allowed to internalize HRV and were then exposed to ozone or nitrogen dioxide. A significantly more than additive effect of both oxidants on virus-induced cytokine release was observed. This was especially pronounced with nitrogen dioxide, which increased IL-8 release by 250 per cent above the sum of either stimulus independently (Spannhake *et al*., 2002). These and similar studies underscore the ability of respiratory epithelial cells to respond powerfully to external stimuli and significantly modulate the pathophysiology of the airway.

14.3 Particulate matter

An extensive series of studies by several groups have shown a strong association between both chronic and short-term exposures to elevated levels of particulate matter (PM) in the ambient environment and increases in morbidity and mortality. These studies have identified this risk in populations residing in areas of environmental character that appear to be diverse in terms of geography, industrial development, and socioeconomic status (e.g. Bell *et al*., 2004; Dockery *et al*., 1993; Dominici *et al*., 2006; Kettunen *et al*., 2007; Nawrot *et al*., 2007; Pope *et al*., 1991; Schwartz and Dockery, 1992a, 1992b; Stolzel *et al*., 2006). A wide range of epidemiologic, controlled human exposure, animal, and *in vitro* studies have been directed toward understanding the nature of PM size and composition that imparts toxicity at the epithelial cell interface. In addition, questions regarding the triggers linking daily increases in PM levels with cardio- and cerebrovascular mortality, and the manner in which these signals might be transduced to result in the observed pathology at target sites distant from the lungs remain to be answered. Clearly, in studies of exposures other than those conducted under controlled laboratory conditions, the confounding effects of ever-present co-pollutants represent a major challenge to identifying those characteristics of ambient PM that are responsible for the observed adverse health effects.

14.3.1 Cell surface activity

Total suspended particles (TSP) in the air can be grouped into three fractions by size (mass median aerodynamic diameter): course (10–2.5 μm), fine ($<$2.5–0.1 μm), and ultrafine ($<$0.1 μm) particles. In some cases, the PM fractions are isolated based on the selected

particle size and contain all smaller-sized particles below that cut-off, as in the case of PM_{10} ($<10\,\mu m$), $PM_{2.5}$ ($<2.5\,\mu m$) and $PM_{0.1}$ ($<0.1\,\mu m$) fractions. Coarse and fine fractions constitute the greatest mass of PM deposited on airway surfaces and have received the most attention in terms of their toxic and pathogenic effects. Ultrafine particulates interact with airway surfaces by means of electrostatic, steric and other adhesive forces and, because of their small size, their interactions with cell membranes and interstitial regions may be very different from larger particles. The potential biological activity of the different fractions can vary considerably as a result of their differing sources and resulting composition. Coarse fractions arise primarily from mechanical sources, whereas fine and ultrafine fractions derive from combustion processes, leading to their high content of nitrates, sulfates, elemental and organic carbon and certain metals. As would be expected, the broad spectrum of active constituents present in and on ambient PM have the potential to activate epithelial cells through a near full range of signalling pathways available within exposed cells. As described below, many of these involve activation of relatively nonspecific oxidant and other stress-related pathways at concentrations that are non-cytotoxic. Furthermore, the interplay between these numerous components of PM that characterizes its multi-factorial stimulus further complicates our understanding of the translation of ambient exposure to potential health effect outcome by airway epithelial cells.

The extent to which PM is taken up into epithelial cells or moves through the airway epithelium to extrapulmonary sites or to the systemic circulation is unclear. Furthermore, the significance of this mechanism in the observed non-respiratory morbidity and mortality in humans following acute PM exposure is controversial. In the case of ultrafine PM, some studies suggest that movement from the airway into lung tissue compartments and to non-pulmonary organs can be identified (Geiser *et al*., 2005; Kreyling *et al*., 2002), although there was no indication that epithelial cell transport played a role in this movement. Although one study reported rapid and substantial movement of technetium 99m ultrafine particles from lungs to the systemic circulation in healthy subjects (Nemmar *et al*. 2002), subsequent investigations by others using the same system indicated the presence of minimal levels of translocation across the epithelium in both healthy subjects and those with COPD (Brown *et al*., 2002, Wiebert *et al*., 2006). Using transmission electron microscopy, investigators have described the time-course of diesel exhaust particle (DEP) endocytosis by human nasal epithelial cells in primary culture (Boland *et al*., 1999). Entry of DEP into the cells was associated with increased release of IL-8, GM-CSF and IL-1β that was attenuated by treatment of the DEP to remove adsorbed organic compounds.

Particles landing on the mucosal surface of the airway become entrapped in a protein- and lipid-rich and aqueous environment into which their soluble constituents may be released. These include biologically-active organic pollutants, acid salts, biological contaminants and metals that can activate epithelial cells in the absence of PM entry into the cells. Polycyclic aromatic hydrocarbons (PAHs) derive from incomplete combustion of organic molecules and are found associated with PM from vehicular (gasoline and diesel), as well as industrial, emissions. Many have been identified as carcinogens, and their ability to form DNA adducts in airway epithelial cells has been described (e.g. Pohjola *et al*., 2003). Components removed from DEP by organic extraction have been shown to elicit responses in normal HBE cells that mimic those of un-extracted DEP and include release of IL-8, GM-CSF and RANTES (Kawasaki *et al*., 2001). Studies have demonstrated that PAHs can be transferred from the surfaces of PM to airway epithelial cell membranes which they then rapidly cross and accumulate in cytosolic lipid vesicles (Mazzarella *et al*., 2007; Penn *et al*., 2005). Depending upon the PM

source, debris and products from bacterial or other infectious agents may also be presented at the epithelial cell surface. Studies have provided evidence that delivery of biological stimuli by this route can trigger responses in primary HBE cells through activation of Toll-like receptors on cell surfaces (Becker *et al*., 2005b, 2005c).

14.3.2 Stimulus transduction and intracellular pathways

Exposure of human and animal airway epithelial cells in culture to ambient $PM_{2.5}$ and residual oil fly ash (ROFA) increased the production of ROS within the cells with resultant activation of NF-κB and expression of pro-inflammatory mediators. This activity was reduced or inhibited by treatment with antioxidants or free radical scavengers (Nam *et al*., 2004; Shukla *et al*., 2000; Stringer and Kobzik, 1998). Immunohistochemical staining of biopsies from healthy subjects exposed to DEP implicated upstream involvement of p38 and JNK mitogen-activated protein (MAP) kinases in activation of transcription factors NF-κB and AP-1 in bronchial epithelial cells, also suggesting the involvement of oxidative stress (Pourazar *et al*., 2005). As pointed out by Nam and colleagues (2004), NF-κB also induces generation of nitric oxide (NO) by mediating expression of inducible nitric oxide synthase (iNOS). Thus, this provides a pathway for increasing pro-inflammatory activity in the airway in addition to those leading to the expression of inflammatory cytokines. In addition to p38 MAP kinase, activation of the extracellular signal-regulated kinase (ERK) has been reported to be necessary for GM-CSF expression in primary HBE cells exposed to an ultrafine/fine ($<0.18\,\mu m$) PM (Reibman *et al*., 2002). In a study of ultrafine synthetic particles of elemental carbon, exposure of HBE cells resulted in IL-8 promoter activity, gene expression and protein release that were not observed to be accompanied by NF-κB binding or promoter activity. However, the effect was associated with a biphasic activation of p38 MAP kinase (Kim *et al*., 2005). These findings suggest that naked carbonaceous ultrafine particles may interact with HBE cell pro-inflammatory processes differently than combustion-derived PM.

A great deal of interest has been generated by the activities of transition metals present in various PM fractions. Particles generated by combustion of coals, oils and oil products, and other organic fuels and that are present in ambient air have been reported to contain a variety of metals including aluminium, arsenic, chromium, copper, iron, lead, manganese, nickel, selenium, silicon, strontium, titanium, vanadium, and zinc. It is likely that seasonal differences in PM metal content – along with other components – play a role in the variation in biological activity reported for samples collected at different locations and at different times of the year in a given location (Becker *et al*., 2005a; Seagrave *et al*., 2006). In early studies, iron associated with urban air particulates was observed to be mobilized in aqueous solution and, when PM was incubated with cells of a human alveolar epithelial line, cellular content of the iron storage protein ferritin was increased, suggesting mobilization of iron from the PM to the cells (Smith and Aust, 1997). It was also demonstrated that synthesis and release of IL-6, IL-8 and TNF_{α} by HBE cells in response to ROFA could be inhibited by addition of a metal chelator or a free radical scavenger (Carter *et al*., 1997). These and other early studies provided the basis for numerous investigations that followed which began to dissect the various roles that metals play in PM-related cardio-respiratory pathology.

Iron-containing particles have been shown to activate NK-κB by a process that includes movement of the metal ions across the cell membrane and upstream involvement of Src

and the epidermal growth factor receptor (EGFR) (Churg *et al*., 2005; Cao *et al*., 2007). Exposure of a human airway epithelial cell line to $zinc^{++}$ induced IL-8 gene and protein expression that was associated with phosphorylation of the AP-1 components c-Fos and c-Jun. In addition, $zinc^{++}$ induced phosphorylation of ERK, JNK and p38 MAPKs, which was required for IL-8 expression, while inhibiting ERK and JNK phosphatase activity (Kim *et al*., 2006). These results suggest that this common PM metal constituent both initiates and protects the upstream signalling pathway for IL-8 expression in exposed cells. Metal—metal interactions within epithelial cells are also apparent. In cultures of rodent airway epithelial cells, combinations of copper and zinc were found to cause greater cell stress and cytotoxicity than either metal individually (Pagan *et al*., 2003). Nickel-sulfate-induced release of IL-8 by a human airway epithelial cell line was abolished by addition of either $iron^{++}$ or $iron^{+++}$ to the cultures. In contrast, the expression of hypoxia-linked genes also induced in the cells by nickel was unaffected by iron co-exposure, suggesting a complex interaction of distinct pathways of activation for these two metals (Salnikow *et al*., 2004). It may be that metal exposures associated with PM can adversely affect epithelial cell host defense mechanisms. A recent study demonstrated that treatment of cells of the human alveolar epithelial line, A549, with ROFA resulted in an inhibition of stimulated expression of human beta-defensin-2 (Klein-Patel *et al*., 2006). This small cationic protein, that represents an important component of pulmonary innate immunity, is secreted into airway surface liquids and has broad antimicrobial activity in the lung.

14.3.3 Stimulation of intercellular signalling

As indicated above, the most commonly observed changes in airway epithelial cell gene expression in response to PM exposure are those involved with oxidant stress-associated and pro-inflammatory pathways. Typical of studies of HBE cells exposed to DEP and ambient PM_{10} and $PM_{2.5}$, is the release of cytokines IL-6, IL-8, GM-CSF and TNFα, with preferential polarity in apical or basolateral release sometimes reported (Auger *et al*., 2006; Becker *et al*., 2005c; Fujii *et al*., 2001; Takizawa *et al*., 2000a). Contributing to PM-initiated movement of inflammatory cells into the epithelium is the intracellular stimulation of the cyclooxygenase (COX)-2 pathway activity and the enhanced expression of amphiregulin, a ligand of the pro-inflammatory EGFR, by HBE cells (Becker *et al*., 2005c; Blanchet *et al*., 2004). These activities initiate *de novo* inflammation in healthy airway and, importantly, have the ability to exacerbate chronic inflammatory states, such as exist in COPD and asthma, potentially causing an acute increase in disease severity. In addition, ambient PM exposure has been reported to induce HBE expression of MIP-3α/CCL20, the ligand for the CCR6 receptor on a subgroup of dendritic cells (Reibman *et al*., 2003). In concert with the effect of ROFA on beta-defensin-2 described above, these data suggest that PM may alter the characteristics of immune responses in airway exhibiting allergen-induced inflammation. However, investigations aimed at assessing these effects by comparing cellular responses to short-term or acute DEP exposures have provided inconsistent results in studies of nasal and lower airway in which responses in asthmatic and non-asthmatic subjects were compared (Holgate *et al*., 2003; Kongerud *et al*., 2006; Stenfors *et al*., 2004). Epidemiologic studies of disease-associated susceptibility, however, point to increased risk of PM exposures with regard to cardiovascular endpoints in individuals with pre-existing conditions such as COPD (e.g. Peel *et al*., 2007; Sunyer and Basagana, 2001).

14.3.4 Associations with respiratory disease

Of special current interest is identification of the signalling pathways through which exposure of the airway epithelial surfaces to PM is translated to acute mortality associated with extra-pulmonary organs, such as the heart and brain. Three principal mechanisms have emerged as likely candidates. These are: (1) alterations in autonomic nervous system control of cardiac function, for example, heart rate variability; (2) increased instability of atherosclerotic plaques or of clotting mechanisms leading to vascular infarction; and (3) effects of pulmonary-derived mediators that induce vascular constriction or directly interfere with cardiac myocyte function.

Several epidemiologic studies have reported an association between short-term exposures to increased levels of PM and decreases in heart rate variability (e.g. Liao *et al*., 2004; Magari *et al*., 2001). However, in one study this effect was dependent upon the locality of the exposure (Timonen *et al*., 2006), perhaps due to differences in PM composition, and in another, no effect on heart rate variability was detected in subjects with or without pre-existent cardiovascular disease (Sullivan *et al*., 2005). Characterization of PM composition was not made in any of these studies and leaves open the possibility that differences in metal or organic constituents may have contributed to these disparate findings. Furthermore, the possible linkage between epithelial signalling and afferent nerve stimulation in the airway accounting for the observed influence on autonomic cardiac control remains unclear.

Airway epithelial cells have been shown to both release and bind endothelin (ET)-1 (Pegorier *et al*., 2006; Ninomiya *et al*., 1995) a peptide with potent vasoconstrictor and cardiac ionotropic actions. Animal studies have shown that ambient PM exposure increases circulating levels of ET leading to vasopressor responses and enhanced ventricular arrhythmia in rats with experimental myocardial infarction (Kang *et al*., 2002; Vincent *et al*., 2001). Release of this mediator from epithelial cells alone or in combination with pulmonary endothelial cells stimulated by the release of IL-8 and vascular endothelial growth factor from epithelial cells may contribute to cardiac instability in individuals exposed to ambient PM (Chauhan *et al*., 2005).

The stability of pre-existing atherosclerotic plaques can be jeopardized by enhanced inflammation in systemic vessels, perhaps resulting from the upregulation of vascular adhesion molecules and the recruitment of leukocytes to sclerotic regions. In animal studies, exposure to PM stimulates the bone marrow and induces the release of PMNs and other leukocytes into the circulation (van Eeden *et al*., 2005). This stimulation was associated with increased circulating levels of IL-1β and IL-6, known to be released by the airway epithelium in response to PM exposure. In addition, it has been proposed that redox-active constituents of combustion-derived particles that transit the epithelium may enhance systemic inflammation and contribute to vascular sclerotic lesion progression and instability leading to infarction (Delfino *et al*., 2005). Studies in apolipoprotein-E-deficient mice suggest that this process may be enhanced by disfunction of lipid metabolism in susceptible individuals (Chen and Nadziejko, 2005).

One of the primary concerns for long-term exposure to PM is for increased lung cancer risk associated with chronic exposure to their pathogenic constituents. Using data collected by the American Cancer Society in their Cancer Prevention II study, Pope and colleagues concluded that lung cancer mortality, in addition to all-cause and cardiopulmonary mortality, was associated with exposure to fine PM (Pope *et al*., 2002). Potential mechanisms leading to this association have been described. One of the first studies was conducted using a

spontaneously derived cell line of alveolar type II epithelial cells exposed to the $PM_{2.5}$ fraction derived from ambient PM_{10} collected on filters (Timblin *et al*., 1998). Exposure of the cells to nontoxic concentrations of $PM_{2.5}$ initiated the c-*jun* kinase signalling pathway leading to c-jun phosphorylation and transcriptional activation of AP-1-regulated genes. These activities were associated with an increased number of cells undergoing DNA synthesis. The observation of this signalling cascade points to similarities between PM exposure and other stimuli that lead to the carcinogenic process in airway epithelial cells.

14.4 Secondhand cigarette smoke

The risks associated with the exposure to mainstream tobacco smoke as a consequence of smoking cigarettes are well known with regard to the development of chronic obstructive pulmonary disease (COPD) and lung cancer. Since the 1986 Surgeon General's Report (Centers for Disease Control, 1986), it has become increasingly well understood that exposure of non-smokers to secondhand smoke (SHS) or sidestream smoke released from burning cigarettes may also pose a significant health risk. Some of the earliest studies indicated that SHS contained markedly higher levels of PAHs than did mainstream smoke (Grimmer *et al*., 1987). Analysis of data recently made available from tobacco company research indicates that, in some tests, SHS appeared to exhibit greater toxicity and tumorigenicity than mainstream smoke (Schick and Glantz, 2005). Data from this research also indicate that, as SHS smoke 'ages' in the indoor air, its toxicity significantly increases (Schick and Glantz, 2006). These data support the notion that exposure to secondhand smoke poses a significant respiratory health hazard in the indoor environment and represents an important and sometimes prevalent air pollutant with which the respiratory epithelial cell lining interacts.

In addition to its PM constituent, which principally falls in the $PM_{2.5}$ ($<2.5\,\mu m$) range, the gas/vapour phase of SHS is rich in alkenes, nonspecific and tobacco-specific nitrosamines, aromatic and heterocyclic hydrocarbons, and amines. In addition, metals, such as cadmium, lead, zinc and arsenic, can be present in varying amounts. Thus, the interaction of SHS with epithelial cells shares many general mechanisms with those of other combustion-derived airborne PM, with differences based largely on the unique range and levels of tobacco-associated organic constituents that are present. The volatile organics compounds (VOCs) derived by combustion from these organic molecules contribute substantially to the toxicity of the gas/vapour phase (Pouli *et al*., 2003). At the present time, the vast majority of information available regarding the interactions of cigarette smoke (CS) with airway epithelial cells comes from studies in which mainstream tobacco smoke or an aqueous cigarette smoke extract (CSE) that contains many of its volatile components, were used. Analysis by microarray of fully differentiated primary human bronchial epithelial cells exposed to mainstream smoke clearly indicates that the activities of many categories of gene pathways are modulated in response to this multifaceted exposure (Maunders *et al*., 2007).

14.4.1 Cell surface activity

Exposure to various forms of CS has been demonstrated to increase fluids at epithelial cell surfaces primarily by interfering with intercellular tight junctions and by stimulating mucus production. The mechanisms through which reversible leakage across the epithelial barrier is regulated in response to non-cytotoxic levels of CS are not fully understood. One recent

study offers evidence that pathways involving Rho kinase and myosin light chain kinase may exhibit opposing activities by which this flux of ions and macromolecules through the junctions can be modulated (Olivera *et al*., 2007).

14.4.2 Stimulus transduction and intracellular pathways

Perhaps the most prominent and generic of the effects of CS on airway epithelial cells is the activation of redox-sensitive pathways. This activation occurs through mechanisms identical, or similar, to those already described for other gaseous and metal-containing PM pollutants. Typically, pathways of activation involve kinase cascades leading to the transactivation of pro-inflammatory genes by transcription factors such as NF-κB and AP-1. As previously described, the consequences of this activity include the release of inflammation-inducing cytokines, chemokines, and lipid mediators into the epithelial mucosa and the enhanced expression of adhesion molecules and receptors on cell surfaces. In addition, the activation of sulfhydryl and other redox-sensitive sensors by CS components results in the stimulation of antioxidant defences. Heme oxygenase-1 and peroxidases, such as GPX2, are two antioxidant enzymes that play a role in epithelial cell protection following CS exposure. A recent study indicates that the extensive expression of GXP2 in lung epithelial cells in response to CS is dependent upon the binding of nuclear factor-E2-related factor (Nrf2) – a transcription factor broadly involved in upregulating antioxidant defenses – to the GXP2 response element in the cells (Singh *et al*., 2006).

Exposure of the airway epithelium to CS leads to transcriptional upregulation of MUC5AC, the predominant mucin in human airway. This effect is thought to be one of several factors that contribute to the pathology of cigarette-induced chronic obstructive pulmonary disease (COPD). In recent years, studies from many laboratories, including that of Carol Basbaum, have shed considerable light on the pathways involved with this aspect of airway stimulation resulting from CS exposure and its relationship to other sources of oxidant stress to the epithelium. It is now believed that reactive oxidant species generated by CS lead to stimulation of ERK and JNK pathways through activation of EGFR and Src, respectively. These activation pathways combine to result in AP-1 mediated MUC5AC expression (Gensch *et al*., 2004). The mucus-rich airway of COPD patients are often infected with bacteria and contain a variety of pro-inflammatory cytokines. When cells of the human airway epithelial line, NCI-H292, were challenged with CSE in addition to bacterial LPS or TNFα, MUC5AC production was synergistically increased. The synergism appeared to be dependent, at least in part, upon the activity of EGFR (Baginski *et al*., 2006). A similar effect on IL-6 release in response to CSE has also been observed (Beisswenger *et al*., 2004).

It has also been shown that CS causes a pronounced epithelial cell upregulation of the protooncoproteins c-Jun, c-Fos and Fra-1 that was linked to the activation of AP-1 (Zhang *et al*., 2005). Data have indicated the overexpression of these AP-1 family members in some epithelial cell cancers. The pathway leading to this upregulation required the activation of EGFR through a mechanism that was dependent upon initiation by matrix metalloproteinase (MMP) activity (Zhang *et al*., 2005). This signalling cascade, with elements similar to those observed in responses to $PM_{2.5}$ exposures and described earlier, may add to a framework for mechanisms of transition from normal epithelium to malignancy under conditions of chronic activation. Long-term exposure to CS may also induce the expression of otherwise silent genes within epithelial cells. UCHL1, a member of the ubiquitin proteasome pathway present only in bronchial neuroendocrine cells of non-smokers, was found to be expressed

in ciliated epithelial cells of smokers. The epithelial cell presence of this enzyme, which is found overexpressed in more than half of all lung cancers, suggests that events associated with this consequence of CS exposure may also contribute to transformational changes in these cells (Carolan *et al*., 2006).

Unique to the chemical composition of CS is the presence of tobacco-specific carcinogenic nitrosamines derived from nicotine, the most potent of which is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Airway epithelial cells express metabolic activities capable of bioactivating NNK, through α-hydroxylation pathways, to produce alkylating agents believed to be primarily responsible for DNA alkylation linked to adenocarcinogenesis (Hoffman and Hecht, 1985). This activity is largely carried out by cytochrome P450 2A13 (Smith *et al*., 2007). NNK has also been demonstrated to stimulate the proliferation of normal bronchial epithelial cells in a process involving increased expression of cyclin D1 protein that is dependent upon NF-κB activation (Ho *et al*., 2005). In addition to the tobacco-specific nitrosamines, a wide range of PAHs with similar potential carcinogenic activities in epithelial cells, such as benzo(a)pyrene, are present in tobacco smoke, as they are in other forms of smoke from incomplete combustion processes.

Under proper conditions of intensity and persistence of activation by components of cigarette smoke and other environmental pollutants, the various pathways of epithelial cell signalling described above can potentially lead to carcinogenic outcomes in exposed cells.

14.4.3 Stimulation of intercellular signalling

The release of pro-inflammatory mediators from airway cells in response to cigarette smoke and its various preparations has been a fairly consistent finding in both *in vivo* and *in vitro* studies. Primary among these are IL-8 and growth-related oncogene (GRO)-alpha, which primarily stimulate neutrophil chemoattraction, and monocyte chemotactic protein (MCP)-1, a principal stimulant of alveolar macrophages. Analysis of bronchoalveolar lavage fluid suggests that IL-8 and GRO-alpha are elevated in the airway of smokers compared to non-smokers, and that their levels can increase acutely following CS exposure. Although the origins of these mediators cannot be determined in lavage studies, these and related data suggest that epithelial cells represent a primary source of chemotactic activity in smokers (Mio *et al*., 1997; Morrison *et al*., 1998; Takizawa *et al*., 2000b).

The vast majority of studies of the effects of CSE, and cigarette smoke condensate (CSC), that contains particulate components, on HBE cells demonstrate increased gene and protein expression of several pro-inflammatory mediators. These include IL-8, IL-6, GRO-α and ICAM-1, with the involvement of NF-κB playing a primary role in their transcriptional activation (Glader *et al*., 2006; Hellermann *et al*., 2002; Kode *et al*., 2006; Mio *et al*., 1997; Richter *et al*., 2002). Receptors for the complement-derived anaphylatoxin C5a (C5aR) are expressed on the surface of HBE cells. Exposure of these cells to CSE in the presence of C5aR ligand can mediate IL-8 release through a PKC-dependent mechanism (Wyatt *et al*., 1999). These and a great number of other studies underscore the broad range of mechanisms through which epithelial cells can make primary contributions to the airway inflammation that accompanies chronic exposure to CS.

In addition to the synthesis of cytokine mediators, evidence indicates that CS-initiated pathways leading to NF-κB activation can also result in the synthesis and release of lipid mediators by the COX-2 pathway (Shishodia *et al*., 2003).

14.4.4 Associations with respiratory disease

Despite the increase in regulations that prohibit smoking indoors at business and other commercial sites in order to reduce SHS exposure, these actions are limited in terms of world-wide impact. Furthermore, because time spent indoors at home is estimated at 40–60 per cent for adults, this unregulated environment is likely to represent the greatest source of SHS in families that contain a smoker, especially for infants and children. The Global Youth Tobacco Surveys that were conducted in 132 countries over the period from 1999 to 2005 indicated that 56 per cent and 44 per cent of students were exposed to SHS in public places and at home, respectively (The GTSS Collaborative Group, 2006).

An association between maternal smoking and reduced lung function in asthmatic children has been described and is now well established. In an early study of asthmatic children from 7 to 17 years of age, measures of FEV_1 and $FEF_{25–75}$ were significantly decreased in those whose mothers were smokers compared to those whose mothers were not (Murray and Morrison, 1986). The inability to distinguish between the contributions of pre- and postnatal exposure in most of these studies was recently addressed by Moshammer *et al.* (2006). In a study of more than 20, 000 children from 6 to 12 years of age living in nine countries of Europe and North America, they were able to identify small, but significant, associations between SHS and both FEV_1 and maximal expiratory flow at 50 per cent of vital capacity (MEF_{50}) in children exposed during the postnatal period only. The significance of this finding is that decreased lung function in early childhood is a known risk factor for the development of wheezing and asthma in children. Childhood exposure to SHS has also been shown to be associated with the incidence of adult asthma, suggesting the potential for latency in the health effects of exposure (Skorge *et al.*, 2005).

In addition to the direct effects of SHS on the respiratory system, evidence has been accumulating that SHS exposure is associated with acute cardiovascular disease (Raupach *et al.*, 2006). Consistent with earlier studies, analysis of data from non-smoking patients with a first event of acute myocardial infarction or unstable angina and non-smoking healthy controls showed an association of disease with ETS exposure. Exposure less than three times per week was associated with a 26 per cent higher risk and regular exposure was associated with a 99 per cent higher risk (Panagiotakos *et al.*, 2002). These findings may reflect similarities with the data showing an association between non-cigarette-smoke-related PM exposures and cardiovascular morbidity and mortality discussed previously. Beyond the evidence supporting the involvement of the specific epithelial cell activities described in sections above in CS-related respiratory pathogenesis, the specific mechanisms through which epithelial cell activity might mediate or contribute to pathology in downstream vessels and organs remains to be determined.

14.5 Conclusions

Epithelial cells of the respiratory tract interact both directly and indirectly with a broad range of airborne environmental gases and particulates. These unique cells have developed an effective defensive battery of protective and responsive pathways to reduce damage to the airway lining and to facilitate recovery from potentially toxic exposures. In most instances, these defenses are adequate to reduce health risk associated with such exposures on an acute or subacute basis, although the system can certainly be overwhelmed in susceptible individuals.

Furthermore, some of the negative consequences of exposure of respiratory epithelial cells to airborne pollutants are known to be initiated or maintained by activities within the cells themselves. This seemingly paradoxical capability of cells that are positioned at the airway interface largely for the purpose of host defense in part reflects the complex consequences of exposures to contemporary agents that were not predominant in the evolutionary environment. Increasing our understanding of the mechanisms through which these detrimental health effects arise will inform strategies to assist epithelial cells in their protective role.

References

Auger F, Gendron MC, Chamot C, Marano F, Dazy AC (2006) Responses of well-differentiated nasal epithelial cells exposed to particles: role of the epithelium in airway inflammation. *Toxicol Appl Pharmacol*, **215**, 285–294.

Avissar NE, Reed CK, Cox C, Frampton MW, Finkelstein JN (2000) Ozone, but not nitrogen dioxide, exposure decreases gluthathione peroxidases in epithelial lining fluid of human lung. *Am J Respir Crit Care Med*, **162**, 1342–1347.

Ayyagari VN, Januszkiewicz A, Nath J (2004) Pro-inflammatory responses of human bronchial epithelial cells to acute nitrogen dioxide exposure. *Toxicology*, **197**, 149–164.

Baginski TK, Dabbagh K, Satiawatcharaphong C, Swinney DC (2006) Cigarette smoke synergistically enhances respiratory mucin induction by proinflammatory stimuli. *Am J Respir Cell Mol Biol*, **35**, 165–174.

Ballinger CA, Cueto R, Squadrito G, Coffin JF, Velsor LW, Pryor WA, Postlethwait EM (2005) Antioxidant-mediated augmentation of ozone-induced membrane oxidation. *Free Radic Biol Med*, **4**, 515–526.

Barnett AG, Williams GM, Schwartz J, Neller AH, Best TL, Petroeschevsky AL, Simpson RW (2005) Air pollution and child respiratory health: a case-crossover study in Australia and New Zealand. *Am J Respir Crit Care Med*, **171**, 1272–1278.

Bayram H, Sapsford RJ, Abdelaziz MM, Khair OA (2001) Effect of ozone and nitrogen dioxide on the release of proinflammatory mediators from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients in vitro. *J Allergy Clin Immunol*, **107**, 287–294.

Bayram H, Rusznak C, Khair OA, Sapsford RJ, Abdelaziz MM (2002) Effect of ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures of non-asthmatic and asthmatic subjects. *Clin Exp Allergy*, **32**, 1285–1292.

Becker S, Dailey LA, Soukup JM, Grambow SC, Devlin RB, Huang YC (2005a) Seasonal variations in air pollution particle-induced inflammatory mediator release and oxidative stress. *Environ Health Perspect*, **113**, 1032–1038.

Becker S, Dailey L, Soukup JM, Silbajoris R, Devlin RB (2005b) TLR-2 is involved in airway epithelial cell response to air pollution particles. *Toxicol Appl Pharmacol*, **203**, 45–52.

Becker S, Mundandhara S, Devlin RB, Madden M (2005c) Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: further mechanistic studies. *Toxicol Appl Pharmacol*, **207**, 269–275.

Beisswenger C, Platz J, Seifart C, Vogelmeier C, Bals R (2004) Exposure of differentiated airway epithelial cells to volatile smoke in vitro. *Respiration*, **71**, 402–409.

Belanger K, Gent JF, Triche EW, Bracken MB, Leaderer BP (2006) Association of indoor nitrogen dioxide exposure with respiratory symptoms in children with asthma. *Am J Respir Crit Care Med*, **173**, 297–303.

Bell ML, Samet JM, Dominici F (2004) Time-series studies of particulate matter. *Annu Rev Public Health*, **25**, 247–280.

Bell ML, Dominici F, Samet JM (2005) A meta-analysis of time-series studies of ozone and mortality with comparison to the national morbidity, mortality, and air pollution study. *Epidemiology*, **16**, 436–445.

Blanchet S, Ramgolam K, Baulig A, Marano F, Baeza-Squiban A (2004) Fine particulate matter induces amphiregulin secretion by bronchial epithelial cells. *Am J Respir Cell Mol Biol*, **30**, 421–427.

Boland S, Baeza-Squiban A, Fournier T, Houcine O, Gendron MC, Chevrier M, Jouvenot G, coste A, Aubier M, Marano F (1999) Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *Am J Physiol*, **276**, L604–613.

Boot JD, de Haas S, Tarasevych S, Roy C, Wang L, Amin D, Cohen J, Sterk PJ, Miller B, Paccaly A, Burggraaf J, Cohen AF, Diamant Z. (2007) Effect of an NK1/NK2 receptor antagonist on airway responses and inflammation to allergen in asthma. *Am J Respir Crit Care Med*, **175**, 450–457.

Bosson J, Stenfors N, Bucht A, Helleday R, Pourazar J, Holgate ST, Kelly FJ, Sandstrom T, Wilson S, Frew AJ, Blomberg A (2003) Ozone-induced bronchial epithelial cytokine expression differs between healthy and asthmatic subjects. *Clin Exp Allergy*, **33**, 777–782.

Broeckaert F, Arsalane K, Hermans C, Bergamaschi E, Brustolin A, Mutti A, Bernard A. (1999) Lung epithelial damage at low concentrations of ambient ozone. *Lancet*, **353**, 900–901.

Brown JS, Zeman KL, Bennett WD (2002) Ultrafine particle deposition and clearance in the healthy and obstructed lung. *Am J Respir Crit Care Med*, **166**, 1240–1247.

Cao D, Tal TL, Graves LM, Gilmour I, Linak W, Reed W, Bromberg PA, Samet JM (2007) Diesel exhaust particulate-induced activation of Stat3 requires activities of EGFR and Src in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, **292**, L422–429.

Carolan BJ, Heguy A, Harvey BG, Leopold PL, Ferris B, Crystal RG (2006) Up-regulation of expression of the ubiquitin carboxyl-terminal hydrolase L1 gene in human airway epithelium of cigarette smokers. *Cancer Res*, **66**, 10729–10740.

Carter JD, Ghio AJ, Samet JM, Devlin RB (1997) Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol Appl Pharmacol*, **146**, 180–188.

Centers for Disease Control (CDC) (1986) 1986 Surgeon General's report: the health consequences of involuntary smoking. *MMWR Morb Mortal Wkly Rep*, **35**, 769–770.

Chauhan AJ, Inskip HM, Linaker CH, Smith S, Schreiber J, Johnston SL, Holgate ST (2003) Personal exposure to nitrogen dioxide (NO2) and the severity of virus-induced asthma in children. *Lancet*, **361**, 1939–1944.

Chauhan V, Breznan D, Thomson E, Karthikeyan S, Vincent R (2005) Effects of ambient air particles on the endothelin system in human pulmonary epithelial cells (A549). *Cell Biol Toxicol*, **21**, 191–205.

Chen LC, Nadziejko C (2005) Effects of subchronic exposures to concentrated ambient particles (CAPS) in mice. V. CAPs exacerbate aortic plaque development in hyperlipidemic mice. *Inhal Toxicol*, **17**, 217–224.

Chen LL, Tager IB, Peden DB, Christian DL, Ferrando RE, Welch BS, Balmes JR (2004) Effect of ozone exposure on airway responses to inhaled allergen in asthmatic subjects. *Chest*, **125**, 2328–2335.

Churg A, Xie C, Wang X, Vincent R, Wang RD (2005) Air pollution particles activate NF-kappaB on contact with airway epithelial cell surfaces. *Toxicol Appl Pharmacol*, **208**, 37–45.

Delfino RJ, Sioutas C, Malik S (2005) Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. *Environ Health Perspect*, **113**, 934–946.

Devlin RB, McConnell WF, Mann R, Becker S, House DE Schreinemachers D, Koren HS (1991) Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am J Respir Cell Mol Biol*, **4**, 72–81.

Dockery DW, Pope CA 3rd, Xu X, Spengler JD, Ware JH, Fay ME, Ferris BG Jr, Speizer FE (1993) An association between air pollution and mortality in six U.S. cities. *N Engl J Med*, **329**, 1753–1759.

Dokic D, Howarth HP (2006) Effects of ozone on the nasal mucosa (epithelial cells). *Prilozi*, **27**, 115–126.

Dominici F, Peng RD, Bell ML, Pham L, McDermott A, Zeger SL, Samet JM (2006) Fine particulate air pollution and hospital admission for cardiovascular and respiratory diseases. *JAMA*, **295**, 1127–1134.

Fanucchi MV, Plopper CG, Evans MJ, Hyde DM, Van Winkle LS, Gershwin LJ, Schelegle ES (2006) Cyclic exposure to ozone alters distal airway development in infant rhesus moneys. *Am J Physiol Lung Cell Mol Physiol*, **291**, L644–650.

Foster WM, Costa DL, Langenback EG. (1987) Ozone exposure alters tracheobronchial mucociliary function in humans. *J Appl Physiol*, **63**, 996–1002.

Frank R, Liu MC, Spannhake EW, Mlynarek S, Macri K, Weinmann GG (2001) Repetitive ozone exposure of young adults: evidence of persistent small airway dysfunction. *Am J Respir Crit Cre Med*, **164**, 1253–1260.

Fujii T, Hayashi S, Hogg JC, Vincent R, Van Eeden SF (2001) Particulate matter induces cytokine expression in human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, **25**, 265–271.

Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J, Gehr P (2005) Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ Health Perspect*, **113**, 1555–1560.

Gensch E, Gallup M, Sucher A, Li D, Gebremichael A, Lemjabbar H, Mengistab A, Dasari V, Hotchkiss J, Harkema J, Basbaum C (2004) Tobacco smoke control of mucin production in lung cells requires oxygen radicals Ap-1 and JNK. *J Biol Chem*, **279**, 39085–39093.

Gerrity TR, Bennett WD, Kehrl H, DeWitt PJ. (1993) Mucociliary clearance of inhaled particles measured at 2 h after ozone exposure in humans. *J Appl Physiol*, **74**, 2984–2989.

Glader P, Moller S, Lilja J, Wieslander E, Lofdahl CG, von Wachenfeldt K (2006) Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. *Respir Med*, **100**, 818–827.

Grimmer G, Naujack KW, Dettbarn G (1987) Gaschromatographic determination of polycyclic aromatic hydrocarbons, aza-arenes, aromatic amines in the particle and vapor phase of mainstream and sidestream smoke of cigarettes. *Toxicol Lett*, **35**, 117–124.

Hanania NA, Tarlo SM, Silverman F, Urch B, Senathirajah N, Zamel N, Corey P (1998) Effect of exposure to low levels of ozone on the response to inhaled allergen in allergic asthmatic patients. *Chest*, **114**, 752–756.

Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS (2002) Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. *Respir Res*, **3**, 22.

Ho YS, Chen CH, Wang YJ, Pestell RG, Albanese C, Chen RJ, Chang MC, Jeng JH, Lin SY, Liang YC, Tseng H, Lee WS, Lin JK, Chu JS, Chen LC, Lee CH, Tso WL, Lai YC, Wu CH (2005) Tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone (NNK) induces cell proliferation in normal human bronchial epithelial cells through NFkappaB activation and cyclin D1 up-regulation. *Toxicol Appl Pharmacol*, **205**, 133–148.

Hoffmann D, Hecht SS (1985) Nicotine-derived N-nitrosamines and tobacco-related cancer: current status and future directions. *Cancer Research*, **45**, 935–944.

Holgate ST, Sandstrom T, Frew AJ, Stenfors N, Nordenhall C, Salvi S, Blomberg A, Helleday R, Soderberg M (2003) Health effects of acute exposure to air pollution. Part I: Healthy and asthmatic subjects exposed to diesel exhaust. *Res Rep Health Eff Inst*, **112**, 1–30.

Holz O, Mücke M, Paasch K, Böhme S, Timm P, Richter K, Magnussen H, Jörres RA (2002) Repeated ozone exposures enhance bronchial allergen responses in subjects with rhinitis or asthma. *Clin Exp Allergy*, **32**, 681–689.

Iles KE, Dickinson DA, Wigley AF, Welty NE, Blank V, Forman HJ (2005) HNE increases HO-1 through activation of the ERK pathway in pulmonary epithelial cells. *Free Radic Biol Med*, **39**, 355–364.

Ito K, De Leon SF, Lippmann M (2005) Associations between ozone and daily mortality: analysis and meta-analysis. *Epidemiology*, **16**, 447–457.

Jaspers I, Chen LC, Flescher E (1998) Inductin of interleukin-8 by ozone is mediated by tyrosine kinase and protein kinase A, but not by protein kinase C. *J Cell Physiol*, **177**, 313–323.

Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M (1998) Lipid ozonation products activate phospholipases A2, C, and D. *Toxicol Appl Pharmacol*, **2**, 338–349.

Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M (1999) Induction of inflammatory mediators in human airway epithelial cells by lipid ozonation products. *Am J Respir Crit Care Med*, **6**, 1934–1942.

Kang YJ, Li Y, Zhou Z, Roberts AM,. Cai L, Myers Sr, Wang L, Schuchke DA (2002) Elevation of serum endothelins and cariotoxicity induced by particulate matter (PM2.5) in rats with acute myocardial infarction. *Cardiovasc Toxicol*, **2**, 253–261.

Kawasaki S, Takizawa H, Takami K, Desaki M, Okazaki H, Kasama T, Kobayashi K, Yamamoto K, Nakahara K, Tanaka M, Sagai M, Ohtoshi T (2001) Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, **24**, 419–426.

Kelly FJ, Tetley TD (1997) Nitrogen dioxide depletes uric acid and ascorbic acid but not glutathione from lung lining fluid. *Biochem J*, **325**, 95–99.

Kermani S, Ben-Jebria A, Ultman JS (2006) Kinetics of ozone reaction with uric acid, ascorbic acid, and glutathione at physiologically relevant conditions. *Arch Biochem Biophys*, **451**, 8–16.

Kettunen J, Lanki T, Tiittanen P, Aalto PP, Koskentalo T, Kulmala M, Salomaa V, Pekkanen J (2007) Associations of fine and ultrafine particulate air pollution with stroke mortality in an area of low air pollution levels. *Stroke*, **38**, 918–922.

Kim YM, Reed W, Lenz AG, Jaspers I, Silbajoris R, Nick HS, Samet JM (2005) Ultrafine carbon particles induce interleukin-8 gene transcription and p38 MAPK activation in normal human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, **288**, L432–441.

Kim YM, Reed W, Wu W, Bromberg PA, Graves LM, Samet JM (2006) Zn2+induced IL-8 expression involves AP-1, JNK, and ERK activities in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, **290**, L1028–1035.

Klein-Patel ME, Diamond G, Boniotto M, Saad S, Ryan LK (2006) Inhibition of beta-defensin gene expression in airway epithelial cells by low doses of residual oil fly ash is mediated by vanadium. *Toxicol Sci*, **92**, 115–125.

Kode A, Yang SR, Rahman I (2006) Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. *Respir Res*, **7**, 132.

Kongerud J, Madden MC, Hazucha M, Peden D (2006) Nasal responses in asthmatic and nonasthmatic subjects following exposure to diesel exhaust particles. *Inhal Toxicol*, **18**, 589–594.

Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A (2002) Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A*, **65**, 1513–1530.

Lang DS, Jorres RA, Mucke M, Siegfried W, Magnussen H (1998) Interactions between human bronchoepithelial cells and lung fibroblasts after ozone exposure in vitro. *Toxicol Lett*, **96/97**, 13–24.

Leikauf GD, Zhao Q, Zhou S, Santrock J (1993) Ozonolysis products of membrane fatty acids activate eicosanoid metabolism in human airway epithelial cells. *Am J Respir Cell Mol Biol*, **9**, 594–602.

Leikauf GD, Simpson LG, Santrock J, Zhao Q, Abbinante-Nissen J, Zhou S, Driscoll KE (1995) Airway epithelial cell responses to ozone injury. *Environ Health Perspect*, **103** (Suppl 2), 91–95.

Liao D, Duan Y, Whitsel EA, Zheng ZJ, Heiss G, Chinchilli VM, Lin HM (2004) Association of higher levels of ambient criteria pollutants with impaired cardiac autonomic control: a population-based study. *Am J Epidemiol*, **159**, 768–777.

Magari SR, Hauser R, Schwartz J, Williams PL, Smith TJ, Christiani DC (2001) Association of heart rate variability with occupational and environmental exposure to particulate air pollution. *Circulation*, **104**, 986–991.

Maunders H, Patwardhan SR, Phillips J, Clack A, Richter A (2007) Human bronchial epithelial cell transcriptome: gene expression changes following acute exposure to whole cigarette smoke in vitro. *Am J Physiol Lung Cell Mol Physiol*, **292**, L1248–1256.

Mazzarella G, Ferraraccio F, Prati MV, Annunziata S, Bianco A, Mezzogiorno A, Liguori G, Angelillo IF, Cazzola M (2007) Effects of diesel exhaust particles on human lung epithelial cells: an in vitro study. *Respir Med*, **49**, 784–791.

McDonnell WF, Kehrl HR, Abdul-Salaam S, Ives PJ, Folinsbee LJ, Devlin RB, O'Neil JJ, Horstman DH (1991) Respiratory responses of humans exposed to low levels of ozone for 6.6 hours. *Arch Environ Health*, **46**, 145–150.

McKinnon KP, Madden MC, Noah TL, Devlin RB (1993) In vitro ozone exposure increases release of arachidonic acid products from a human bronchial epithelial cell line. *Toxicol Appl Pharmacol*, **118**, 215–223.

Menzel, DB (1976) The role of free radicals in the toxicity of air pollutants (nitrogen oxides and ozone). In *Free Radicals in Biology* (9th edn), Pryor WA (ed.), Academic Press, New York, pp. 181–202.

Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI (1997) Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med*, **155**, 1770–1776.

Morrison D, Strieter RM, Donnelly SC, Burdick MD, Kunkel SL, MacNee W (1998) Neutrophil chemokines in bronchoalveolar lavage fluid and leukocyte-conditioned medium from nonsmokers and smokers. *Eur Respir J*, **12**, 1067–1072.

Morrison D, Rahman I, Macnee W (2006) Permeability, inflammation and oxidant status in airspace epithelium exposed to ozone. *Respir Med*, **100**, 2227–2234.

Moshammer H, Hoek G, Luttmann-Gibson H, Neuberger MA, Antova T, Gehring U, Hruba F, Pattenden S, Rudnai P, Slachtova H, Zlotkowska R, Fletcher T (2006) Parental smoking and lung function in children: an international study. *Am J Respir Crit Care Med*, **173**, 1255–1263.

Mudway IS, Kelly FJ (1998) Modeling the interactions of ozone with pulmonary epithelial lining fluid antioxidants. *Toxicol Appl Pharmacol*, **148**, 91–100.

Murray AB, Morrison BJ (1986) The effect of cigarette smoke from the mother on bronchial responsiveness and severity of symptoms in children with asthma. *J Allergy Clin Immunol*, **77**, 575–581.

Mustafa, MG (1990) Biochemical basis of ozone toxicity. *Free Radic Biol Med*, **9**, 245–265.

Nam HY, Choi BH, Lee JY, Lee SG, Kim YH, Lee KH, Yoon HK, Song JS, Kim HJ, Lim Y (2004) The role of nitric oxide in the particulate matter (PM2.5)-induced NFkappaB activation in lung epithelial cells. *Toxicol Lett*, **148**, 95–102.

Nawrot TS, Torfs R, Fierens F, De Henauw S, Hoet PH, Van Kersschaever G, De Backer G, Nemery B (2007) *J Epidemiol Community Health*, **61**, 146–149.

Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B (2002) Passage of inhaled particles into the blood circulation in humans. *Circulation*, **105**, 411–414.

Nichols BG, Woods JS, Luchtel DL, Corral J, Koenig JQ (2001) Effects of ozone exposure on nuclear factor-kappaB activation and tumor necrosis factor-alpha expression in human nasal epithelial cells. *Toxicol Sci*, **60**, 356–362.

Ninomiya H, Yu XY, Uchida Y, Hasegawa S, Spannhake EW (1995) Specific binding of entothelin-1 to canine tracheal epithelial cells in culture. *Am J Physiol*, **268**, L424–431.

Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C (2007) Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. *Inhal Toxicol*, **19**, 13–22.

Overton JH, Miller FJ (1988) Dosimetry modeling of inhaled toxic reactive gases. In *Air Pollution, the Automobile and Public Health*, Watson AY, Bates RR and Kennedy D (eds), Washington, DC, pp. 367–385.

Pagan, I, Costa DL, McGee JK, Richards JH, Dye JA (2003) Metals mimic airway epithelial injury induced by in vitro exposure to Utah Valley ambient particulate matter extracts. *J Toxicol Environ Health A*, **66**, 1087–1112.

Panagiotakos DB, Chrysohoou C, Pitsavos C, Papaioannou I, Skoumas J, Stefanadis C, Toutouzas P (2002) The association between secondhand smoke and the risk of developing acute coronary syndromes, among non-smokers, under the presence of several cardiovascular risk factors: the CARDIO2000 case-control study. *BMC Public Health*, **2**, 9.

Pathmanathan S, Krishna MT, Blomberg A, Helleday R, Kelly FJ, Sandstrom T, Holgate ST, Wilson SJ, Frew AJ (2003) Repeated daily exposure to 2 ppm nitrogen dioxide upregulates the expression of IL-5, IL-10, IL-13, and ICAM-1 in the bronchial epithelium of healthy human airways. *Occup Environ Med*, **60**, 892–896.

Peel JL, Metzger KB, Klein M, Flanders WD, Mulholland JA, Tolbert PE (2007) Ambient air pollution and cardiovascular emergency department visits in potentially sensitive groups. *Am J Epidemiol*, **165**, 625–633.

Pegorier S, Wagner LA, Gleich GJ, Pretolani M (2006) Eosinophil-derived cationic proteins activate the synthesis of remodeling factors by airway epithelial cells. *J Immunol*, **177**, 4861–4869.

Penn A, Murphy G, Barker S, Henk W, Penn L (2005) Combustion-derived ultrafine particles transport organic toxicants to target respiratory cells. *Environ Health Perspect*, **113**, 956–963.

Phillips TJ, Bloudoff DP, Jenkins PL, Stroud KR (1999) Ozone emissions from a 'personal air purifier'. *J Expo Anal Environ Epidemiol*, **9**, 594–601.

Pohjola SK, Lappi M, Honkanen M, Rantanen L, Savela K (2003) DNA binding of polycyclic aromatic hydrocarbons in a human bronchial epithelial cell line treated with diesel and gasoline particulate extracts and benzo[a]pyrene. *Mutagenesis*, **18**, 429–438.

Pope CA 3rd, Dockery DW, Spengler JD, Raizenne ME (1991) Respiratory health and PM10 pollution. A daily time series analysis. *Am Rev Respir Dis*, **144**, 668–674.

Pope CA 3rd, Burnett RT, Thun MJ, Calle EE, Krewski D, Ito K, Thurston GD (2002) Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *JAMA*, **287**, 1132–1141.

Postlethwait EM, Cueto R, Velsor LW, Pryor WA (1998) O3-induced formation of bioactive lipids: estimated surface concentrations and lining layer effects. *Am J Physiol*, **6**, L1006–1016.

Pouli AE, Hatzinikolaou DG, Piperi C, Stavridou A, Psallidopoulos MC, Stavrides JC (2003) The cytotoxic effect of volatile organic compounds of the gas phase of cigarette smoke on lung epithelial cells. *Free Radic Biol Med*, **34**, 345–355.

Pourazar J, Mudway IS, Samet JM, Helleday R, Blomberg A, Wilson SJ, Frew AJ, Kelly FJ, Sandstrom T (2005) Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways. *Am J Physiol Lung Cell Mol Physiol*, **289**, L724–730.

Pryor WA (1992) How far does ozone penetrate into the pulmonary air/tissue boundary before it reacts? *Free Radic Biol Med*, **12**, 83–88.

Pryor WA, Church DF (1991) Aldehydes, hydrogen peroxide, and organic radicals as mediators of ozone toxicity. *Free Radic Biol Med*, **11**, 41–46.

Pryor WA, Houk KN, Foote CS, Fukuto JM, Ignarro LJ, Squadrito GL, Davies KJ (2006) Free radical biology and medicine: it's a gas, man! *Am J Physiol Regul Integr Comp Physiol*, **3**, R491–511.

Pulfer MK, Taube C, Gelfand E, Murphy RC (2005) Ozone exposure in vivo and formation of biologically active oxysterols in the lung. *J Pharmacol Exp Ther*, **312**, 256–264.

Raupach T, Schafer K, Konstantinides S, Andreas S (2006) Secondhand smoke as an acute threat for the cardiovascular system: a change in paradigm. *Eur Heart J*, **27**, 386–392.

Reibman J, Hsu Y, Chen LC, Kumar A, Su WC, COI W, Talbot A, Gordon T (2002) Size fractions of ambient particulate matter induce granulocyte macrophage colony-stimulating factor in human bronchial epithelial cells by mitogen-activated protein kinase pathways. *Am J Respir Cell Mol Biol*, **27**, 455–462.

Reibman J, Hsu Y, Chen LC, Bleck B, Gordon T (2003) Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol*, **28**, 648–654.

Richter A, O'Donnell RA, Powell RM, Sanders MW, Holgate ST, Djukanovic R, Davies DE (2002) Autocrine ligands for the epidermal growth factor receptor mediate interleukin-8 release from bronchial epithelial cells in response to cigarette smoke. *Am J Respir Cell Mol Biol*, **27**, 85–90.

Rombout PH, Dormans JA, Marra M, van Esch GJ (1986) Influence of exposure regimen on nitrogen dioxide-induced morphological changes in the rat lung. *Environ Res*, **41**, 466–480.

Sajjan US, Jia Y, Newcomb DC, Bentley JK, Lukacs NW, LiPuma JJ, Hershenson MB (2006) *H. influenzae* potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 tnd TLR3 expression. *FASEB J*, **20**, 2121–2123.

Salnikow K, Li X, Lippmann M (2004) Effect of nickel and iron co-exposure on human lung cells. *Toxicol Appl Pharmacol*, **196**, 258–265.

Salvi S, Semper A, Blomberg A, Holloway J, Jaffar Z, Papi A, Teran L, Polosa R, Kelly F, Sandstrom T, Holgate S, Frew A (1999) Interleukin-5 production by human airway epithelial cells. *Am J Respir Cell Mol Biol*, **20**, 984–991.

Schelegle ES, Miller LA, Gershwin LJ, Fanucchi MV, Van Winkle LS, Gerriets JE, Walby WF, Mitchell V, Tarkington BK, Wong VJ, Baker GL, Pantle LM, Joad JP, Pinkerton KE, Wu R, Evans MJ, Hyde DM, Plopper CG (2003) Repeated episodes of ozone inhalation amplifies the effects of allergen sensitization and inhalation on airway immune and structural development in Rhesus monkeys. *Toxicol Appl Pharmacol*, **191**, 74–85.

Schick S, Glantz S (2005) Philip Morris toxicological experiments with fresh sidestream smoke: more toxic than mainstream smoke. *Tob Control*, **14**, 396–404.

Schick S, Glantz SA (2006) Sidestream cigarette smoke toxicity increases with aging and exposure duration. *Tob Control*, **15**, 424–429.

Schwartz J, Dockery DW (1992a) Particulate air pollution and daily mortality in Steubenville, Ohio. *Am J Epidemiol*, **135**, 12–19.

Schwartz J, Dockery DW (1992b) Increased mortality in Philadelphia associated with daily air pollution concentrations. *Am Rev Respir Dis*, **145**, 600–604.

Seagrave J, McDonald JD, Bedrick E, Edgerton ES, Gigliotti AP, Jansen JJ, Ke L, Naeher LP, Seilkop SK, Zheng M, Mauderly JL (2006) Lung toxicity of ambient particulate matter from southeastern U.S. sites with different contributing sources: relationships between composition and effects. *Environ Health Perspect*, **114**, 1387–1393.

Shishodia S, Potdar P, Gairola CG, Aggarwal BB (2003) Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF-kappaB activation through inhibition of IkappaBalpha kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. *Carcinogenesis*, **24**, 1269–1279.

Shukla A, Timblin C, BeruBe K, Gordon T, McKinney W, Driscoll K, Vacek P, Mossman BT (2000) Inhaled particulate matter causes expression of nuclear factor (NF)-kappaB-related genes and oxidant-dependent NF-kappaB activation in vitro. *Am J Respir Cell Mol Biol*, **23**, 182–187.

Singh A, Rangasamy T, Thimmulappa RK, Lee H, Osburn WO, Brigelius-Flohe R, Kensler TW, Yamamoto M, Biswal S (2006) Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs is regulated by Nrf2. *Am J Respir Cell Mol Biol*, **35**, 639–650.

Skorge TD, Eagan TM, Eide GE, Gulsvik A, Bakke PS (2005) The adult incidence of asthma and respiratory symptoms by passive smoking in uterus or in childhood. *Am J Respir Crit Care Med*, **172**, 61–66.

Smith BD, Sanders JL, Porubsky PR, Lushington GH, Stout CD, Scott EE (2007) Structure of the human lung cytochrome P450 2A13. *J Biol Chem*, **282**, 17306–17313.

Smith KR, Aust AE (1997) Mobilization of iron from urban particulates leads to generation of reactive oxygen species in vitro and induction of ferritin synthesis in human lung epithelial cells. *Chem Res Toxicol*, **10**, 828–834.

Spannhake EW, Reddy SP, Jacoby DB, Yu XY, Saatian B, Tian J (2002) Synergism between rhinovirus infection and oxidant pollutant exposure enhances airway epithelial cell cytokine production. *Environ Health Perspect*, **110**, 665–670.

Stenfors N, Nordenhall C, Salvi SS, Mudway I, Soderberg M, Blomberg A, Helleday R, Levin JO, Holgate St, Kelly FJ, Frew AJ, Sandstrom T (2004) Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. *Eur Respir J*, **23**, 82–86.

Stolzel M, Breitner S, Cyrys J, Pitz M, Wolke G, Kreyling W, Heinrich J, Wichmann HE, Peters A (2006) Daily mortality and particulate matter in different size classes in Erfurt, Germany. *J Expo Sci Environ Epidemiol*, **17**, 458–467.

Stringer B, Kobzik L (1998) Environmental particulate-mediated cytokine production in lung epithelial cells (A549): role of preexisting inflammation and oxidant stress. *J Toxicol Environ Health A*, **55**, 31–44.

Sullivan JH, Schreuder AB, Trenga CA, Liu SL, Larson TV, Koenig JQ, Kaufman JD (2005) Association between short term exposure to fine particulate matter and heart rate variability in older subjects with and without heart disease. *Thorax*, **60**, 462–466.

Sunyer J, Basagana X (2001) Particles and not gases, are associated with the risk of death in patients with chronic obstructive pulmonary disease. *Int J Epidemiol*, **30**, 1138–1140.

Tager IB, Balmes J, Lurmann F, Ngo L, Alcorn S, Kunzli N (2005) Chronic exposure to ambient ozone and lung function in young adults. *Epidemiology*, **6**, 751–759.

Takizawa H, Ohtoshi T, Kawasaki S, Abe S, Sugawara I, Nakahara K, Matsushima K, Kudoh S (2000a) Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: a review. *Respirology*, **5**, 197–203.

Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Umeda A (2000b) Increased expression of inflammatory mediators in small-airway epithelium from tobacco smokers. *Am J Physiol Lung Cell Mol Physiol*, **278**, L906–913.

The GTSS Collaborative Group (2006) A cross country comparison of exposure to secondhand smoke among youth. *Tob Control*, **15** (Suppl 2), 114–119.

Timblin C, BeruBe K, Churg A, Driscoll K, Gordon T, Hemenway D, Walsh E, Cummins AB, Vacek P, Mossman B (1998) Ambient particulate matter causes activation of the c-jun kinase/stress-activated protein kinase cascade and DNA synthesis in lung epithelial cells. *Cancer Res*, **58**, 4543–4547.

Timonen KL, Vanninen E, de Hartog J, Ibald-Mulli A, Brunekreef B, Gold DR, Heinrich J, Hoek G, Lanki T, Peters A, Tarkiainen T, Tiittanen P, Kreyling W, Pekkanen J (2006), Effects of ultrafine and fine particulate and gaseous air pollution on cardiac autonomic control in subjects with coronary artery disease: the ULTRA study. *J Expo Sci Environ Epidemiol*, **16**, 332–341.

Uppu RM, Pryor WA (1994) The reactions of ozone with proteins and unsaturated fatty acids in reverse micelles. *Chem Res Toxicol*, **7**, 47–55.

van Bree L, Dormans J, Koren HS, Devlin RB, Rombout PJ (2002) Attenuation and recovery of pulmonary injury in rats following short-term, repeated daily exposure to ozone. *Inhal Toxicol*, **14**, 883–900.

Van Eeden SF, Yeung A, Quinlam K, Hogg JC (2005) Systemic response to ambient particulate matter: relevance to chronic obstructive pulmonary disease. *Proc Am Thorac Soc*, **2**, 61–67.

Van Strien RT, Gent JF, Belanger K, Triche E, Bracken MB, Leaderer BP (2004) Exposure to NO2 and nitrous acid and respiratory symptoms in the first year of life. *Epidemiology*, **15**, 471–478.

Vincent R, Kumarathasan P, Geogan P, Bjarnason SG, Guenette J, Berube D, Adamson IY, Desjardins S, Burnett RT, Miller FJ, Battistini B (2001) Inhalation toxicology of urban ambient particulate matter: acute cardiovascular effects in rats. *Res Rep Health Eff Inst*, **104**, 5–54.

Voter KZ, Whitin JC, Torres A, Morrow PE, Cox C, Tsai Y, Utell MJ, Frampton MW (2001) Ozone exposure and the production of reactive oxygen species by bronchoalveolar cells in humans. *Inhal Toxicol*, **13**, 465–483.

Wiebert P, Sanchez-Crespo A, Seitz J, Falk R, Philipson K, Kreyling WG, Moller W, Sommerer K, Larsson S, Svartengren M (2006) Negligible clearance of ultrafine particles retained in healthy and affected human lungs. *Eur Respir J*, **28**, 286–290.

Wyatt TA, Heires AJ, Sanderson SD, Floreani AA (1999) Protein kinase C activation is required for cigarette smoke-enhanced C5a-mediated release of interleukin-8 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, **21**, 283–288.

Yu XY, Takahashi N, Croxton TL, Spannhake EW (1994) Modulation of bronchial epithelial cell barrier function by in vitro ozone exposure. *Environ Health Perspect*, **103** (Suppl 2), 77–89.

Yu, XY, Undem BJ, Spannhake EW (1996) Protective effect of substance P on permeability of airway epithelial cells in culture. *Am J Physiol*, **271**, L889–895.

Zhang Q, Adiseshaiah P, Reddy SP (2005) Matrix metalloproteinase/epidermal growth factor receptor/mitogen-activated protein kinase signaling regulate fra-1 induction by cigarette smoke in lung epithelial cells. *Am J Respir Cell Mol Biol*, **32**, 72–81.

This page intentionally left blank

15

Interactions between Allergens and the Airway Epithelium

Clive Robinson and **Jihui Zhang**[1], **Geoffrey A. Stewart**[2]

[1] *Division of Basic Medical Sciences, St George's, University of London, UK*
[2] *School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, Crawley, Western Australia*

15.1 The airway epithelium as a key target of allergens

The airway epithelium is the ultimate line of physical defence against inhaled materials of diverse origin, composition and biological threat (Gehr and Heyder, 2000). In defending the host, the cells that comprise the airway mucosa act in conjunction with mucus and the soluble components of airway surface liquid that function as essential components of innate immunity. In fulfilling this defensive role, the airway epithelium orchestrates a sub-epithelial network of cells that are responsible for the cohesion and integrity of host defence (Robinson, 1995). Allergens are amongst the many types of inhaled hazard to which the airway epithelium is exposed. In this chapter, we consider the potential interactions of allergens with the airway epithelium and current evidence suggesting that interactions at the mucosal surface are central to the initiation and maintenance of allergy.

Allergens are the, usually proteinaceous, causative factors and triggers for IgE-mediated hypersensitivity reactions (Stewart and Robinson, 2003). In the airway, the most significant allergic conditions are rhinitis and asthma. A good understanding now surrounds the effector mechanisms involved in these conditions, but less is known about how allergens actually initiate and cause allergy (Stewart, 2000). The factors relevant to causation include the characteristics of allergen exposure, the presence of genetic predisposing factors, and the physicochemical and biochemical nature of the allergens themselves.

Considerations of what makes an allergen an allergen and why allergy only develops in some people have led to proposals that non-immunological variables may have significant influence on this outcome (Aas, 1978). One factor suggested is the intrinsic permeability of tissues. If, for example, the airway epithelium was abnormally 'leaky' in an individual, this

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

might increase the probability of allergens contacting the immune system, a process which would be influenced by physicochemical factors such as molecular size. However, this view presupposes the existence of a permeability barrier dysfunction and implies that allergens are otherwise passive threats in the absence of a defect that grants access to the immune system. As will be discussed later, whilst the possible genetic programming of a barrier defect may be important in some conditions, other evidence suggests that permeability may be influenced directly by some allergens.

Attempts to understand allergenicity at the molecular level have focused on searches for common structural motifs within allergens that could explain their ability to elicit allergy (Aalberse and Stadler, 2006; Aalberse, 2006, 2000). With the benefit of hindsight, it is easy to see that allergen diversity makes it unlikely that a single structural motif could provide a general explanation of allergenicity, although immunologically cross-reactive allergens must show some similarity (Aalberse and Stadler, 2006). An alternative perspective on the question of what makes an allergen has come from two converging approaches. One of these has considered the problem in terms of understanding what must happen for allergens to establish contact with antigen presenting cells, a process in which epithelial barriers must have a significant role (Robinson *et al*., 1997; Wan *et al*., 1999). The other has sought to understand the behaviour of allergens through an appreciation of their biological properties that extend beyond molecular shape and a description of their antigenic epitopes (Stewart and Robinson, 2003). Both of these approaches assume, however, that the properties of allergens must be viewed in the context of other factors, such as genetic predisposition and viral infection, which may be complicit in determining, if not the outcome of allergen exposure, the threshold level of allergen required to initiate allergic sensitization.

15.2 The epithelial barrier

As described elsewhere in this volume, the airway epithelium functions as a highly regulated barrier. The sophistication of its function delicately balances the need to protect the host against noxious agents entering from the outside world with a requirement for the regulated physiological movement of cells and solutes into the lumen. Collectively, the cellular structure and its associated functions provide key elements of the immunological, biochemical and physical defence of the airway.

In human airway, the major antigen-presenting cells are dendritic cells (DCs) (Holt *et al*., 1990; Holt, 2002; Lambrecht and Hammad, 2003; Huh *et al*., 2003; van Rijt and Lambrecht, 2005). Secondary mechanisms of antigen presentation exist and include macrophages, inducible bronchus-associated lymphoid tissue and epithelial cells (Delventhal *et al*., 1992; Pabst and Gehrke, 1990; Salik *et al*., 1999), but current evidence suggests that, in human airway, these antigen presentation pathways are either normally less significant or result in the development of immunological tolerance (Ritz *et al*., 2000; Salik *et al*., 1999; Kalb *et al*., 1991; Mezzetti *et al*., 1991).

DCs form a continuous network beneath the airway epithelium, with the dendrites of individual cells arborizing extensively to enable antigen sampling to occur over a large area of mucosa (Holt *et al*., 1990). Although a subset of DCs may protrude lumenally (Jahnsen *et al*., 2006), DCs generally have a predominantly sub-epithelial localization, which means that the probabilities of allergen detection, presentation and antibody-driven responses are likely to be increased when the epithelial barrier reacts to enhance the likelihood of

interactions between allergens and DCs (Herbert *et al*., 1990; Robinson *et al*., 1997; Wan *et al*., 1999). Epithelium-associated DCs are normally unable to stimulate naive T-cells and thus a further feature of the response to an allergen is the provision of signal(s) that enable DCs to mature as they process allergen and migrate to draining lymph nodes to recruit T-cells (van Rijt and Lambrecht, 2005).

At the simplest level, the airway epithelium may be considered as forming a barrier which offers two potential routes for substances to cross it, one transcellular and the other, paracellular. Transcellular movement occurs passively in accordance with the law of diffusion for substances that are sufficiently lipid-soluble to enter the epithelial cell membrane, and which are eventually capable of leaving it down a concentration gradient. In the absence of a purely passive transcellular movement capability, the existence of specific transporters (active or facilitated) or endocytosis might provide other transit mechanisms and, in these cases, proteasomal or lysosomal degradation may result. Paracellular movement can occur passively through the narrow intercellular channels that are formed by the abutment of epithelial cells, but diffusional capacity is low compared to that potentially available for diffusion across the larger surface area of cell membranes. However, for substances unable to move transcellularly, transepithelial passage through paracellular spaces is the only alternative. Significantly, in the context of antigen presentation, the paracellular route provides the possibility of increased direct contact with the dendrites of antigen-presenting cells. Paracellular channels show discrimination in the nature and magnitude of the movement that can occur through them; properties which are conferred by the presence of interepithelial tight junctions (TJs) (Tsukita *et al*., 2001; Citi, 1993). For any macromolecular solute, the paracellular permeability is inversely related to its Stokes' radius because of the size limitation of the channel. For low molecular weight anions and cations the permeability and selectivity of the channels are defined by their composition of transmembrane adhesion proteins (Tsukita *et al*., 2001; Anderson *et al*., 1993; Anderson and Van Itallie, 1995).

Tight junctions are assembled from proteins that create adhesive macromolecular strands which encircle the apical pole of epithelial cells, establishing the barrier properties of the polarized epithelium (Chapter 2). Transmembrane adhesion proteins include occludin (Furuse *et al*., 1993), the claudin family (Furuse *et al*., 1998) and junction-associated molecules (JAMs) (Mandell and Parkos, 2005; Aurrand-Lions *et al*., 2000; Ebnet *et al*., 2000), with occludin and claudins having particularly important roles in adhesion and permeability (Van Itallie and Anderson, 1997; Furuse *et al*., 1998; Balda *et al*., 1996, 2000; Aijaz *et al*., 2006).

Claudins are adhesive proteins of $\sim$ 23 kDa with four transmembrane spans (4TM). They create the distinctive anastomosing strands characteristic of TJs in freeze-fracture replicas of epithelia, and appear to underlie the development of transepithelial electrical resistance and ionic selectivity (Tsukita *et al*., 2001; Furuse *et al*., 1998; Hou *et al*., 2006). Numerous claudins have now been identified, the extracellular domains of which show regions of appreciable sequence homology suggesting conservation of function, but significant differences also exist and confer particular properties on the different family members (Tsukita *et al*., 2001; Furuse *et al*., 1999, 2001; Aijaz *et al*., 2006). This provides some explanation for the considerable variation in transepithelial electrical resistance of epithelia in different parts of the body because the profile of claudins found within any epithelium dictates its electrical resistance and ionic permselectivity (Furuse *et al*., 2001; Hou *et al*., 2006). Occludin may be more significant in governing the paracellular permeability of solutes other than mineral ions and the creation of a transepithelial electrical resistance (Balda *et al*., 1996). Like the claudins,

occludin, and its splice variant occludin-2, are 4TM proteins, but occludin (~65 kDa) is larger than the claudin proteins and has a longer intracellular C-terminal domain (Furuse *et al*., 1993). The first of its two extracellular domains is rich in Tyr and Gly residues, whereas the composition of the second domain is more diverse. Assembly of occludin and claudins into the TJ complex involves interaction of their C-termini with ZO-1, ZO-2 and ZO-3, a group of membrane-associated guanylate kinase-like proteins (MAGUKs) that are key components of TJ plaques (Fanning *et al*., 1998; Furuse *et al*., 1994; Anderson and Van Itallie, 1995; Tsukita *et al*., 2001; Aijaz *et al*., 2006). JAMs differ from other TJ transmembrane proteins (Mandell and Parkos, 2005). They have a single membrane-spanning domain and two immunoglobulin-like domains. Compared to occludin and claudins, their role in TJs is less clear, but like the 4TM proteins, JAMs are capable of binding to cytoplasmic TJ proteins (Bazzoni *et al*., 2000).

15.3 Peptidases and epithelial cell signalling

Epithelial cells are exposed to a variety of proteolytic enzymes in the course of their normal functions, but how these cells respond is not well understood. Physiologically, this process is carefully controlled to prevent dysfunction arising from unregulated proteolysis. Control exists at several levels, including the presence of endogenous inhibitors and by the continued tight binding of a proteolytic enzyme's own prodomain after enzyme maturation. However, if these regulatory mechanisms are overwhelmed or evaded then pathophysiological consequences may evolve.

Epithelial cells express receptors that respond to a variety of peptidases. These receptors, known as protease-activated receptors (PARs), are members of the superfamily of G-protein coupled receptors (GPCRs) and their activation may result in a diverse collection of effects (Coughlin, 2000; Vergnolle *et al*., 2001). The enzymes that activate these receptors cleave the N-terminus of the latent receptor, thereby creating a receptor with a truncated extracellular N-terminus which is able to bind and activate the receptor as a 'tethered ligand'. Although classically activated by serine peptidases such as thrombin (PAR-1, -3 and -4), trypsin (PAR-2, -4) and tryptase (PAR-2), members of the PAR family may also be cleaved by other peptidase classes with activity on the C-terminal side of Lys and Arg. However, if cleavage occurs at different residues the effect may lead to sub-maximal activation, or even inactivation of the receptor.

The signal transduction pathways of PARs involve, at least in part, an increase in phosphoinositide turnover and an increase in intracellular calcium (Berger *et al*., 2001; Schechter *et al*., 1998; Ubl *et al*., 2002). Downstream transduction also involves mitogen-activated protein kinase (MAPK) signalling (Camerer *et al*., 2002; Wang *et al*., 2002; Temkin *et al*., 2002), a series of pathways crucial to the progression of inflammation from initiation to resolution. The net result of MAPK signalling is the phosphorylation of a variety of cytoplasmic and nuclear proteins, alteration of gene expression and the promotion of cell growth, differentiation, apoptosis, inflammation and adaptation to stress (Tibbles and Woodgett, 1999; Waetzig and Schreiber, 2003). The three major groups of MAPKs are the extracellular signal-related kinases (ERKs), the c-jun N-terminal kinases (JNKs) and the p38 kinases. Activation of these groups of MAPKs is controlled by upstream MAP kinases (MAP2Ks) which are hierarchically regulated by families of MAPK kinase kinase (MAP3Ks) that respond to extracellular stimuli through kinase-linked receptors and GPCRs in the cell membrane.

15.4 The biochemical properties of allergens and their contribution to allergenicity

Traditionally, attempts to understand allergenicity have focused on the characterization of the immunological epitopes which allergens display. These are created by linear sequence and by protein conformation, so other studies of allergenicity have naturally examined the possibility that allergens may possess shared structural features. While of undoubted importance, neither epitope analyses nor molecular topology alone provides a satisfactory general understanding of allergenicity. Allergens are highly diverse, and a conundrum of allergenicity is that some of them are clearly more significant than others, for reasons that are not simply explained by their relative environmental abundance or their immunogenicity in experimental tests of lymphocyte responsiveness. This has led to an examination of what other properties of allergens contribute to their allergenicity.

One of the most recently studied aspects of allergenicity is the contribution made by an allergen's biochemical properties, a topic that is beyond the scope of this chapter but which is reviewed in detail (Stewart and Robinson, 2003). The most extensively studied property is enzymatic activity, and in particular the ability to degrade proteins and peptides. Although numerous allergens in the plant kingdom are enzymes, peptidases are most significantly represented in the allergen repertoires of mites, fungi and stinging insects. A striking feature of peptidase allergens is their potency as sensitizers and the high prevalence of immunoreactivity associated with them in allergic patients. House dust mites (HDMs) are amongst the most significant of domestic allergens, and at least four types of peptidase allergen are present within the matrix of inhaled substances associated with HDM allergy. Peptidases may be encountered in the working environment and are usually products of bacteria, fungi or plants. These are important causes of occupational asthma, most notably in the detergent industry where the potency of these materials as sensitizers was recognized almost 40 years ago. These general observations suggest that peptidase activity could be important for the initiation of allergy (Robinson *et al*., 1997). Conversely, allergens lacking peptidase activity are notoriously poor at inducing allergic sensitization. Ovalbumin, one of the most widely used experimental tools in models of allergy, exemplifies well the difficulty in obtaining allergic reactions to non-peptidase antigens. When administered to the airway, ovalbumin typically induces tolerance (Kheradmand *et al*., 2002). Robust IgE-mediated responses are only obtained if ovalbumin is administered subcutaneously or intraperitoneally in the presence of a suitable adjuvant. This suggests that, for inhaled allergens, an essential component of allergenicity is a profile of biological activity that augments allergen delivery across the airway mucosa to DCs, evades the mechanisms that drive tolerance and stimulates processing via Th2 polarized immune responses. There are grounds to believe that peptidase activity provides significant insights into this biological profile and an understanding of events that are essential to both the initiation and maintenance of allergy (Robinson *et al*., 1997; Wan *et al*., 1999; Herbert *et al*., 1995; Ghaemmaghami *et al*., 2002; Kheradmand *et al*., 2002).

Several independent lines of evidence support the view that peptidase activity makes a significant contribution to allergic sensitization. For example, in mice sensitized subcutaneously with glutathione transferase from *Schistosoma mansoni*, the HDM allergen, Der p 1, causes the resulting immune response to develop with Th2 polarity, but when immunization is performed using Der p 1 inactivated by heat, or by the cysteine peptidase inhibitor E-64, the development of Th2 polarity is blunted (Comoy *et al*., 1998). Other studies have established

that inhibition of the enzymatic activity of Der p 1 or papain blunts the development of IgE responses to both Der p 1 itself, and to bystander allergens such as ovalbumin (Chambers *et al*., 1998; Gough *et al*., 1999, 2001, 2003). A similar picture exists for allergic responses to *Aspergillus fumigatus* (Kurup *et al*., 2002). For example, mixed allergens from *A. fumigatus* or *A. oryzae* have a striking effect on intranasal responses to ovalbumin. The normal response to intranasally delivered ovalbumin is tolerance, but this is converted to a strong allergic response by the *Aspergillus* extracts (Kheradmand *et al*., 2002). Pretreatment of the *Aspergillus* extracts with serine peptidase inhibitors diminishes the magnitude of allergic sensitization, suggesting that serine peptidase activity within the extracts plays an essential role in modulating the immunological response (Kheradmand *et al*., 2002). The target(s) inactivated by the inhibitors have not been identified, but several *Aspergillus* allergens are serine peptidases with actions on relevant biological targets (e.g. Asp f 13, Asp f 15, Asp f 18 and Asp o 13) (Tai *et al*., 2006).

Although it remains to be established whether peptidases present in other allergen mixtures (e.g. pollens, insects etc.) behave similarly, a provocative new parallel to the highly successful Linnean method of allergen classification is suggested by the observations with allergens of mites and fungi. This parallel approach transcends classification by source or immunological similarity and divides allergens into those, such as certain HDM and fungal allergens, that have the capability to induce allergy without the need for adjuvants and those, like ovalbumin, that are wholly reliant on the action of other factors to promote sensitization. HDMs and *Aspergillus* species are sources of enzymes other than peptidases (e.g. Asp f 1 ribonuclease, Asp f 9 endoglucanase, Der p 4 amylase and Der p 18 chitinase) and it remains to be established what role these enzyme activities play in sensitization. Similarly, interesting questions are raised about the role of enzymes in pollen allergy, where known peptidase allergens are few in number and enzymatic activity is exemplified by pectate lyase and polygalacturonase, and other types of allergen are prevalent (Stewart and Robinson, 2003). The IgE-independent actions of these enzymes in promoting allergy are yet to be determined in detail. It should also be borne in mind that the influence of a peptidase on sensitization need not be encoded in a molecule which itself is strongly allergenic and in such a situation peptidase activity may be considered as acting principally in an adjuvant capacity. Thus, the numerous serine and cysteine peptidase components of pollen extracts which, while not associated with any known allergen, may be adjuvants in pollen allergy (Bagarozzi and Travis, 1998; Bagarozzi *et al*., 1996, 1998; Cortes *et al*., 2006; Grobe *et al*., 2002, 1999; Hassim *et al*., 1998; Matheson and Travis, 1998; Radlowski, 2005; Raftery *et al*., 2003). Conversely, there is speculation that non-peptidase materials such as endotoxin and unmethylated DNA may behave as adjuvants to peptidase allergens in dust mite allergy (Platts-Mills, 2007).

As is evident from Tables 15.1–15.3, peptidases from numerous sources are known allergens, and these are encountered domestically and/or occupationally. Of the domestic respiratory allergens, those of mite origin are clinically most important and consequently the most studied. This is especially true for Der p 1, the archetype of Group 1 mite allergens and a ‘concept template’ for understanding the significance of peptidase activity related to allergenicity. Group 1 mite allergens are globally the most important of domestic allergens and a therapeutic target, either for specific immunotherapy or for emerging new approaches to allergy treatment and prevention.

Der p 1, in common with other Group 1 mite allergens such as Der f 1 and Eur m 1, is a member of the C1 family of clan CA cysteine peptidases (Robinson *et al*., 1997). In mites, these powerful enzymes digest the resilient structural proteins in their diet (Colloff and Stewart, 1997). These enzymes are initially synthesized as zymogens with an 80 residue

Table 15.1 Known proteolytic allergens from fungal sources

Allergen (source and identity)	Frequency of reactivity (%)	Molecular weight (kDa)	Function
Ascomycota			
Aspergillus fumigatus			
Asp f 5	74	40	Metallopeptidase
Asp f 10	3	34	Aspartic peptidase
Asp f 13	>60	34	Alkaline serine peptidase
Asp f 15	?	16	Homologous with serine peptidase from *Coccidioides immitis*
Asp f 18	79	34	Vacuolar serine peptidase
Cladosporium herbarium			
Cla h 9	?	?	Vacuolar serine peptidase
Penicillium chrysogenum/notatum			
Pen ch 13	>80	34	Alkaline serine peptidase
Pen ch 18	>80	28−34	Vacuolar serine peptidase
Penicillium oxalicum			
Pen o 18	80	34	Vacuolar serine peptidase
Penicillium citrinum			
Pen c 13	100	33	Alkaline serine peptidase
Candida albicans			
Acid peptidase	75	35	Aspartate peptidase
Trichophyton tonsurans			
Tri t 2	42	30	Subtilisin-like peptidase homologous with Pen ch 13 and Pen c 13
Tri t 4	61	83	Dipeptidyl peptidase
Trichophyton rubrum			
Tri r 1/2	?	30	Subtilisin-like peptidase homologous with Pen ch 13 and Pen c 13
Tri r 4	?	83	Dipeptidyl peptidase
Rhodoturala mucilaginosa			
Rho m 2	57	31	Vacuolar serine peptidase

Table 15.2 Known proteolytic allergens from mite aeroallergens

Allergen (source and identity)	Frequency of reactivity (%)	Molecular weight (kDa)	Function
Pyroglyphidae, Glycyphagidae, Acaridae, and Echimyopodidae			
Group 1 (e.g. Der p 1)	>90	25	Cysteine peptidase
Group 3 (e.g. Der p 3)	90	25	Trypsin-like serine peptidase
Group 6 (e.g. Der p 6)	39	25	Chymotrypsin-like serine peptidase
Group 9 (e.g. Der p 9)	>90	28	Collagenase-like serine peptidase

Table 15.3 Known proteolytic aeroallergens associated with occupational allergy

Allergen (source and identity)	Frequency of reactivity (%)	Molecular weight (kDa)	Function
Fungal allergens			
Aspergillus oryzae			
Asp o 13	?	34	Alkaline serine peptidase; subtilase homologue
Cryphonectira parasitica			
Renin	?	34	Aspartate peptidase; shows homology with mammalian and cockroach pepsins
Bacterial allergens			
Bacillus subtilis			
Alcalase	>50	28	Subtilisin serine peptidase
Bacillus licheniformis			
Esperase	>50	28	Subtilisin serine peptidase
Clostridium histolyticum			
Collagenase	>50	68–125	Metallopeptidase
Streptomyces grisens			
	?	36	Chymotrypsin-like serine peptidase
Caricaceae			
Carica papaya (Pawpaw)			
Car p 1	?	23	Papain, cysteine peptidase
Actinidia chinensis (Kiwi fruit)			
Act c 1	100	30	Actinidin, cysteine peptidase
Bromelaceae			
Ananas comosus (Pineapple)			
Ana c 2	?	23	Bromelain, cysteine peptidase
Mammalian peptidases			
Trypsin (porcine)	?	24	Serine peptidase; homologous with mite groups 3,6, and 9 allergens
Chymotrypsin (bovine)	?	25	Serine peptidase; homologous with mite groups 3,6, and 9 allergens
Pepsin (porcine)	?	35	Aspartate peptidase; shows homology with cockroach Bla g 2

propiece that must be removed for the allergens to become enzymatically active and for them to become fully immunogenic. A high degree of similarity exists between the Group 1 mite allergens, a finding of potential therapeutic importance. Although they are related to the C1 family archetype, papain, it is clear that major differences exist between them and, significantly, between the mite allergens and mammalian C1 family enzymes such as the cathepsins. Some key differences concern the binding pockets in the active site of the enzyme, a fact established from the recently obtained crystal structure of recombinant Der p 1 (Figure 15.1).

Mite allergens of Groups 3, 6 and 9 are all serine peptidases with tryptic (Group 3), chymotryptic (Group 6) or collagenolytic (Group 9) specificity. The clinical significance of these allergens is probably less than the Group 1 allergens, reflecting their lower abundance and specific activity within the complete mix of mite peptidase allergens. They have been of particular interest in the context of understanding the molecular recognition of

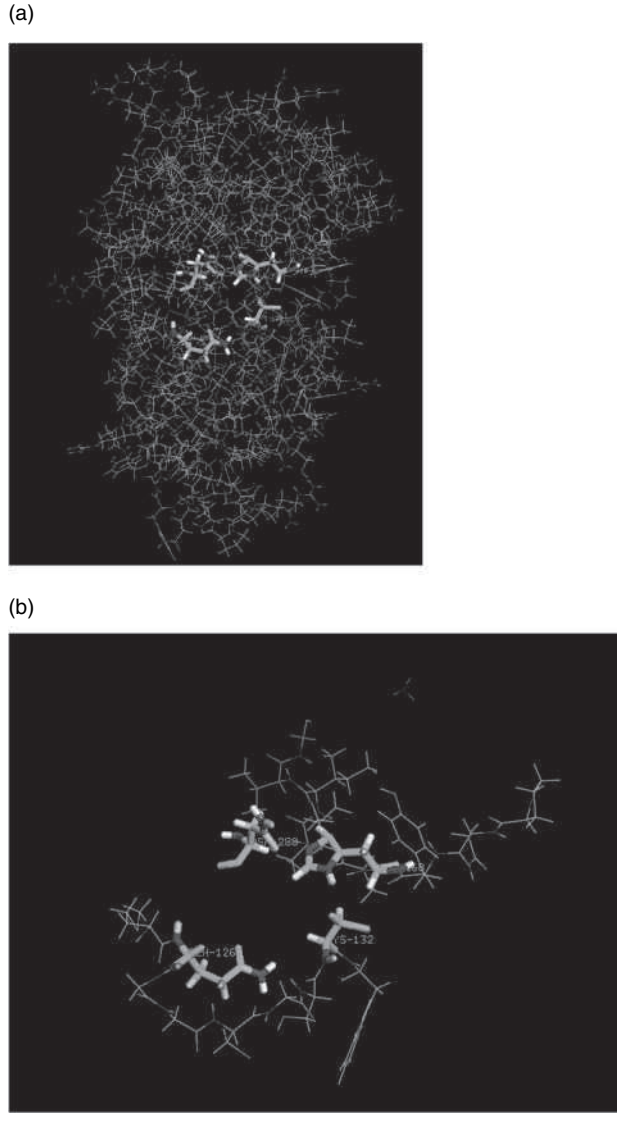


Figure 15.1 (a) Line representation of Der p 1 structure determined by X-ray crystallography with catalytic site residues (Gln126, Cys132, His268, Asn288, numbering from full length sequence) indicated in stick form. (b) Active site residues of Der p 1 showing creation of binding pocket

peptidase allergens in the airway epithelium because their cleavage specificity suggests they activate PARs.

Peptidase activity is also well represented amongst important fungal allergens and an increasing body of work has demonstrated that allergens with serine peptidase activity make important contributions to allergenicity. As with the mite allergens, understanding the effects of the fungal serine peptidases on the airway epithelium is providing valuable new insights into mechanisms of allergy.

15.5 Peptidase contributions to allergic sensitization via the epithelium

Much, if not all, of the potency of peptidases as allergens appears to reside in their ability to execute two general actions: augmentation of the delivery of allergens to DCs and the creation of a signalling environment that fosters the development of allergy, especially in those with a genetic predisposition.

15.5.1 Epithelial permeability

As already described, transepithelial delivery is a necessary step in increasing the probability of allergen detection by DCs, and is one that must be recapitulated for allergic sensitization to be maintained. Despite the significance of this step, interactions between allergens and the epithelial lining of the respiratory tract are not well understood. The largest body of available evidence, appropriately in view of their status as the most significant cause of domestic allergies, concerns HDM allergens.

Evidence for the intraepithelial uptake of HDM allergens is equivocal, with evidence of little (Wan, Zhang and Robinson, unpublished observations) or more significant amounts (Mori *et al*., 1995). A diminished processing of Der p 1 has been reported in airway epithelial cells from people with asthma, although the reasons for this, the mechanisms involved, and the potential significance of the deficit are unknown (Mori *et al*., 1995). However, paracellular routes of allergen delivery are of special interest because they allow a simple and direct route to DCs (Wan *et al*., 1999). HDM allergens would normally be expected to have little access to paracellular channels because these are occluded apically by TJs. However, peptidase allergens have a significant effect on the structure and functional cohesion of TJs. This has been demonstrated for natural mixtures of HDM allergens and also for purified peptidase allergens from HDM (Wan *et al*., 1999, 2000a, 2000b, 2001). The effect of Der p 1 on epithelial permeability was first reported in 1990 (Herbert *et al*., 1990), and subsequently demonstrated in whole airway and in intact sheets of airway mucosa (Herbert *et al*., 1995). The next major advance was the identification of the molecular mechanism and elucidation that TJs are the major target for Der p 1 in the augmentation of epithelial permeability (Wan *et al*. 1999, 2000a). These discoveries were facilitated by technical advances which revealed the high potency of these allergens as localized and reversible disruptors of epithelial permeability (Wan *et al*. 1999, 2000a). With these techniques, it has become clear that significant changes can be induced with the contents of very few faecal pellets (Wan *et al*., 1999). Figure 15.2 shows that TJ cleavage affects cytoplasmic (ZO-1) components in addition to the transmembrane adhesion proteins occludin and claudin-1. The extracellular domains of the adhesion proteins contain cleavage sites for Der p 1 and the

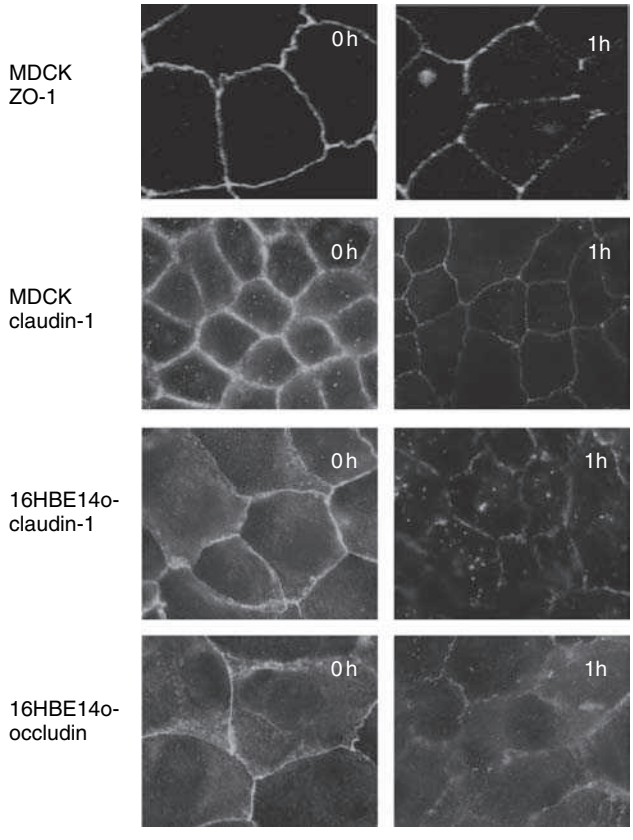


Figure 15.2 Time-dependent disruption of TJs in epithelial monolayers by Der p 1. MDCK or 16HBE14o- human airway epithelial cells were exposed to Der p 1 and fluorescent antibody labelling of TJ proteins performed. After 1 h exposure to Der p 1 disruption of staining of both intracellular (ZO-1) and transmembrane adhesion proteins (occludin, claudin-1) is seen

serine peptidase allergens, consistent with events observed in imaging data (Figures 15.3 and 15.4) (Wan *et al*., 1999, 2000a, 2001). It is also clear that this extracellular protein cleavage is associated with the activation of intracellular protein processing, because one of the major fragments of occludin generated during this process arises by a cleavage within the intracellular C-terminal domain (Wan *et al*., 1999). Two photon imaging and quantitative analysis of reconstructed images reveals the effect of the peptidase allergens on the appearance of TJ rings visualized by fluorescent antibody labelling (Figure 15.5) (Wan *et al*., 1999, 2000a, 2001). An important feature to note is that while these ultrasensitive imaging techniques reveal that the areas immediately surrounding the contact with HDM faecal pellets show profound changes in the appearance of TJs, these frequently exist within larger areas where little disruption is observable. The effect of the peptidase allergens on epithelial cohesion is therefore subtler than the gross derangements in the appearance of the epithelium that accompany chronic persistent asthma or that are typical of the post mortem pathology of patients who died in *status asthmaticus* (Dunnill, 1971, 1960; Naylor, 1962;

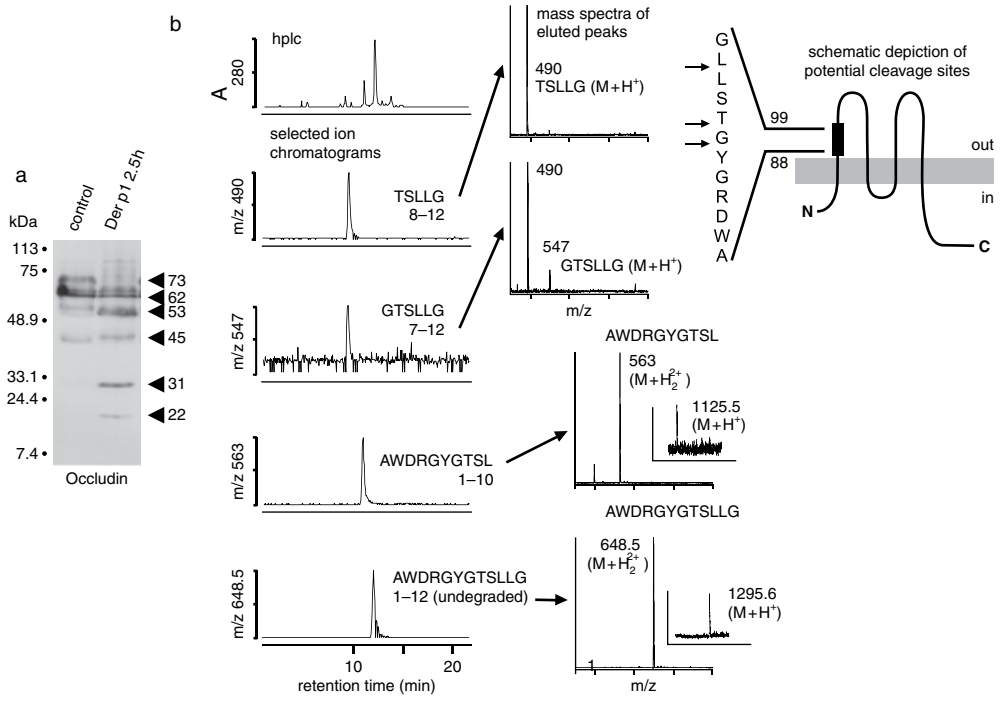


Figure 15.3 (a) Immunoblots of occludin from 16HBE14o- cells prepared from sham-treated cells and after a 2.5-hour exposure to Der p 1. (b) Degradation by Der p 1 of peptide ^{88}AWDRGYGTSLLG^{99} (single letter amino acid code) corresponding to residues 88–99 of human occludin (top right), with identified cleavage sites marked by arrows. The left section of (b) shows the HPLC A_{280} chromatogram and selected ion chromatograms for the 4-h incubation products of Der p 1 with the peptide. Mass spectra for the peptides are shown. Major components correspond to unchanged 12-mer sequences ($M + H^+$, m/z 1295.6; $M + H_2^{2+}$, m/z 648.5) and residues 1–10 ($M + H^+$, m/z 1125.5; $M + H_2^{2+}$, m/z 563), 7–12 ($M + H_+$, m/z 547), and 8–12 ($M + H^+$, m/z 490)

Piacentini *et al*., 1998). The effect of peptidase allergens on TJs produces a non-selective increase in permeability (Figure 15.6(a) and (b)), and inhibition of Der p 1 proteolytic activity prevents both the changes in TJs and the transepithelial delivery of Der p 1 (Figure 15.6(c)). The nonspecificity of the permeability increase means that the transepithelial delivery of any allergen would be increased after TJ cleavage, thus facilitating the contact between DCs and non-peptidase allergens which are unable to cross the epithelium directly. More recent studies have suggested that fungal serine peptidase allergens exert similar effects on TJs (Tai *et al*., 2006).

Repair of TJs after allergen exposure occurs over a time span of a few hours (Wan *et al*., 1999). The repair is a highly ordered process in which rings of ZO-1 are initially reinstated, creating the necessary reticular framework into which the adhesion proteins are then assembled to re-establish junctional integrity (Figure 15.7 (a) and (b)). In more extreme circumstances, caused both by prolonged or highly concentrated exposures to allergen, TJs may disappear entirely (Figure 15.5) and this is associated with changes in desmosomal labelling. The purpose and mechanism of this change in desmosomes is unknown, but

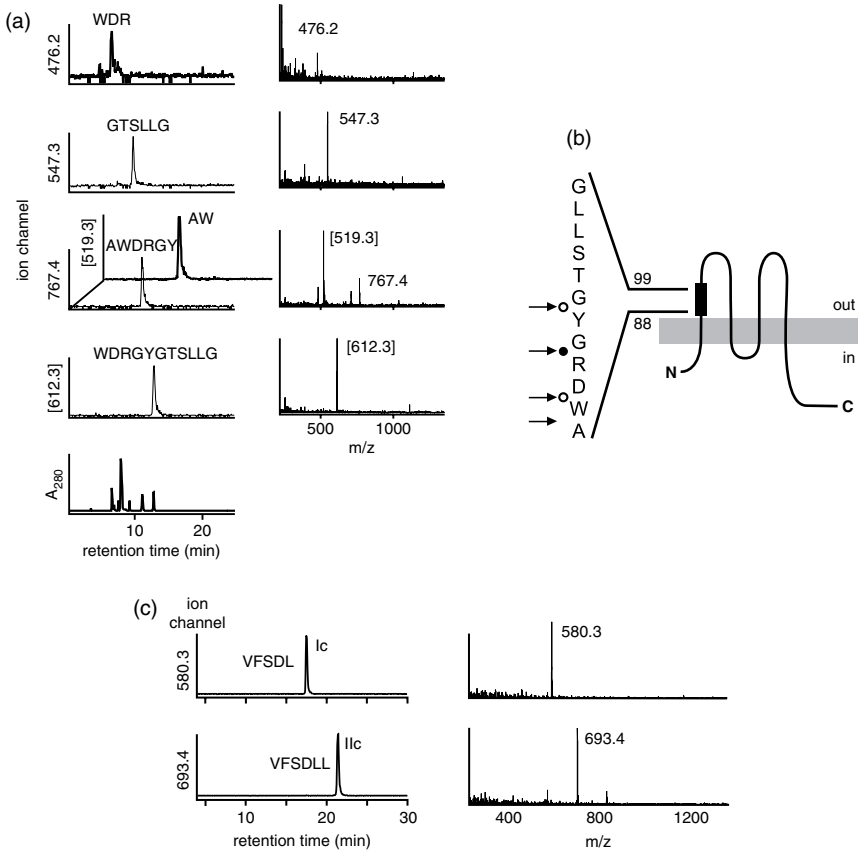


Figure 15.4 (a) HPLC ion chromatograms and electrospray mass spectra of selected fragments following the degradation of occludin extracellular loop peptide (^{88}AWDRGYGTSLLG^{99}) by HDM serine peptidases, Der p 3, Der p 6 and Der p 9. A_{280} ultraviolet (UV) absorbing fragments include WDRGYGTSLLG (13 min, doubly charged ion at *m/z* 612.3), AWDRGY (11.2 min, $M + H^+$ *m/z* 767), DRGYGTSLLG (11.1 min, doubly charged ion at *m/z* 519.3), and GTSLLG ($M + H^+$ *m/z* 547.3) which does not exhibit UV absorbance. Doubly charged ions are bracketed. (b) Diagrammatic representation of HDMFP serine peptidase cleavage sites in relation to the predicted membrane topography of occludin. Arrows denote cleavage sites found in synthetic peptide, filled and open circles denote theoretical cuts for enzymes with tryptic and chymotryptic specificity, respectively. (c) and (d) HPLC-electrospray mass spectrometry showing effects of HDM serine peptidase allergens on claudin-1 peptide KVFDSLLNLNS (c), and WYGNRIVQ (d). Selected ion chromatograms are shown on the left, with the corresponding mass spectra on the right. Note that doubly charged ions are bracketed. Two major fragments of KVFDSLLNLNS are VFDSL (peak Ic, $M + H^+$ *m/z* 580.3) and VFDSLL (peak IIc, $M + H^+$ *m/z* 693.4). WYGNRIVQ was digested into a number of major fragments including YGNRIVQ (peak Id, $M + H^+$ *m/z* 849.5), WYGN (IId, $M + H^+$ *m/z* 539.2), and WYGNR (IIId, $M + H^+$ *m/z* 695.3). Doubly charged ions are bracketed. Free tryptophan (not shown) and unchanged WYGNRIVQ (IVd, $M + H^+$ *m/z* 1035.5) were also present. (e) Shows diagrammatically the cleavage sites in relation to the predicted topography of claudin-1. Arrows denote cleavage sites in synthetic peptide, filled and open circles denote theoretical cuts for enzymes having tryptic and chymotryptic specificity, respectively

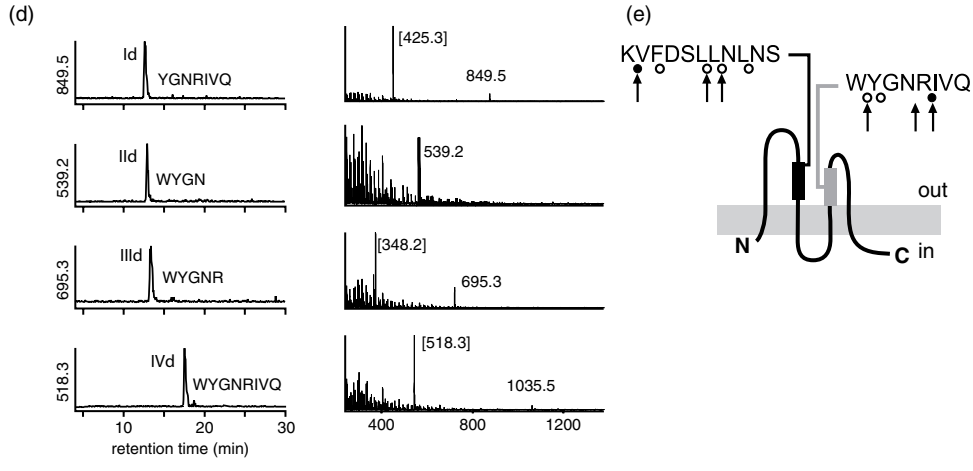


Figure 15.4 (Continued)

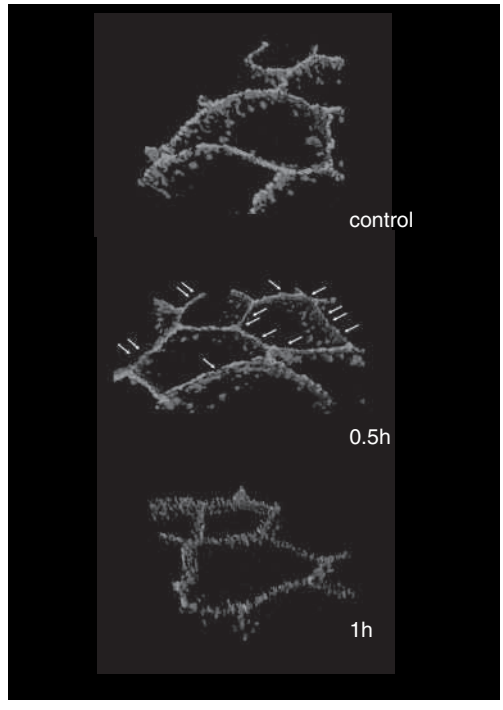


Figure 15.5 Three-dimensional isosurface reconstructions of changes in epithelial adhesions following exposure to HDM allergen. Fluorescent antibody labelling of TJs (green staining) and desmosomes (red staining) was performed and images captured by two-photon molecular excitation microscopy were rendered into 3-D isosurfaces using IRIS Explorer. White arrows show the appearance of initial discontinuities in TJ immunostaining

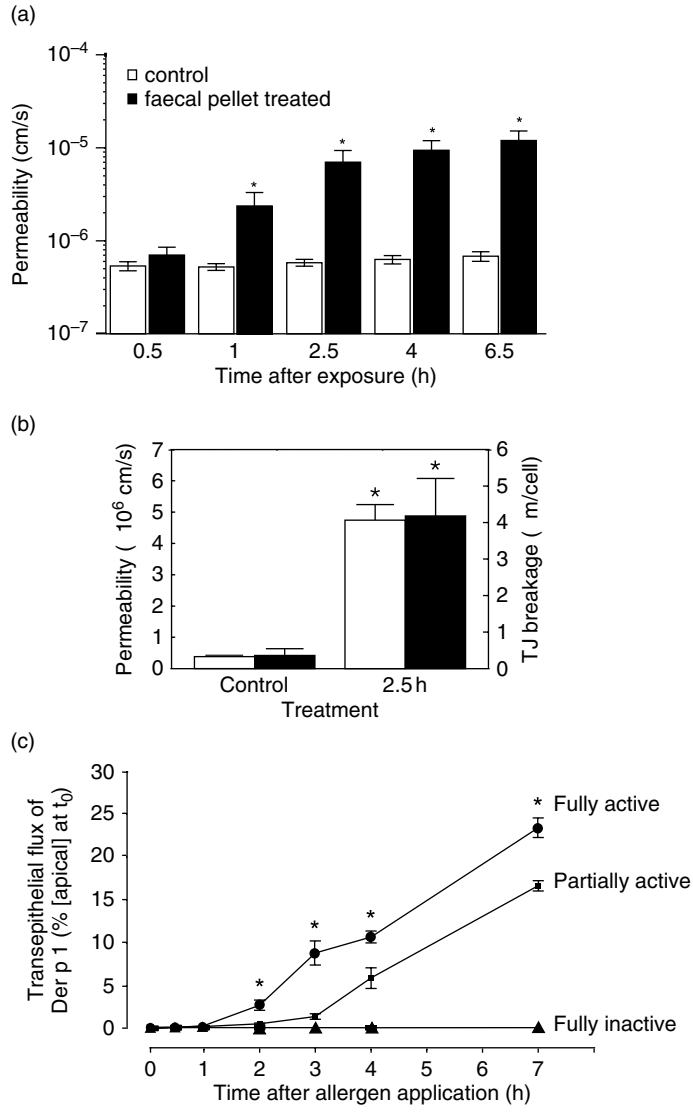


Figure 15.6 (a) Time-dependent effects of HDM faecal pellets (HDMFPs) on the mannitol permeability of MDCK cell monolayers cultured in Transwells. Forty HDMFPs solubilized in EMEM containing 0.5 mM of reduced glutathione were added to monolayers (filled bars). Open bars show monolayers sham treated with EMEM/glutathione alone. *Significant differences from sham-treated monolayers ($P < 0.05$). (b) Effects of Der p 1 on permeability of MDCK cell monolayers showing concordance between changes in [^{14}C]mannitol permeability (open bars) and TJ breakage (i.e. the total length of breaks per cell; filled bars) in the same cell monolayer (*$P < 0.05$). (c) The transepithelial movement of immunoreactive Der p 1 across MDCK cell monolayers depends upon enzymatic activity. Data are expressed as the percentage of the starting concentration ([apical] t_0) of Der p 1 in the apical compartment that was detected in the basal chamber at the indicated time points. Monolayers were treated with allergen that had full catalytic activity, or which was rendered partially active or fully inactive *Responses significantly different from both of the other treatments ($P < 0.05$)

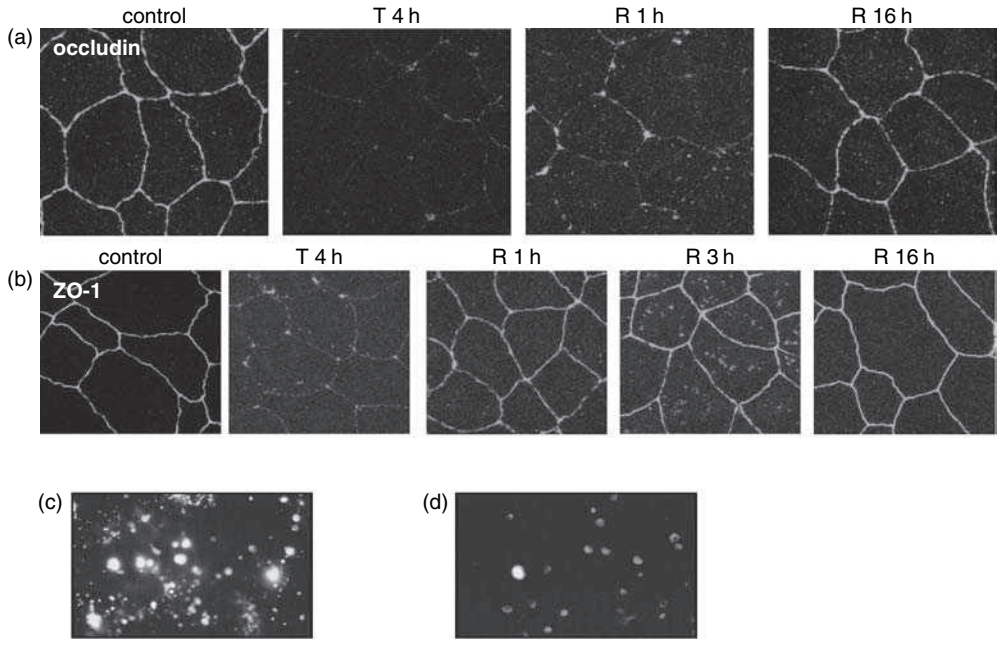


Figure 15.7 (a) and (b) Recovery of occludin and ZO-1 in 16HBE14o- epithelial cell monolayers following exposure to Der p 1. Cells were sham exposed (control) or treated with allergen for 4 hours (T 4 h), and then allowed to recover over 16 h (R 1 h, R 3 h, R 16 h). Images are shown as extended-focus x–y sections. (c) and (d) Apoptosis induced by Der p 1 revealed by annexin V/propidium iodide staining of 16HBE14o- and MDCK cells respectively

because they function as intercellular 'spot welds' and regulate epithelial cohesion (Garrod, 1993; Green and Jones, 1996) it may represent an attempt to increase mechanical resilience in an area of stress.

15.5.2 Apoptosis

For cells unable to maintain anchorage following exposure to peptidase allergens the fate is death by apoptosis (Figure 15.7(c) and (d)) (Baker *et al*., 2003). Apoptosis is known to be triggered separately from TJ cleavage because peptidase allergens can cause apoptosis in cell lines derived from the airway epithelium that constitutively lack TJs (Baker *et al*., 2003). Furthermore, in cell lines that normally express TJs, apoptosis is not affected when the TJs are removed by a calcium switch procedure (Baker *et al*., 2003). The precise trigger for apoptosis caused by peptidase allergens remains unknown, but a component of the initiation or response mechanism(s) probably involves disruption of hemidesmosomal adhesion, causing detachment of cells from the substratum biomatrix. Whether this cell detachment adds to the local increase in epithelial permeability caused by TJ cleavage is unclear. The consequences of apoptotic cell loss may be contained by rapid reformation of intercellular adhesions and a dynamic response of remaining cells to fill the vacancy created by a detached cell. However, if cell detachment is extensive, or if TJ integrity in remaining cells is highly compromised, then apoptosis may contribute to epithelial permeability as has

been demonstrated in gastrointestinal epithelium (Schulzke *et al*., 2006). Although apoptosis is activated separately from TJ cleavage, it has at least one feature which is reminiscent of the latter. In epithelial cells rendered apoptotic by staurosporine (Bojarski *et al*., 2004), the C-terminus of occludin undergoes intracellular cleavage similar to the response evoked by dust mite peptidase allergens (Wan *et al*., 1999).

15.5.3 Receptor cleavage and mediator release

Peptidase allergens initiate a number of IgE-independent pro-inflammatory events, especially the release or activation of mediators. The range of mediators involved encompasses lipids (eicosanoids), proteins (cytokines and chemokines), complement and, in mast cells, histamine (Asokananthan *et al*., 2002b; Kauffman *et al*., 2000; Machado *et al*., 1996; Tai *et al*., 2006; Tomee *et al*. 1997, 1998; King *et al*., 1998). In addition to mediator release, other studies have revealed the potential for peptidase allergens to interact with, and inactivate, other aspects of mucosal defence (Kalsheker *et al*., 1996). The predictable effect of this combination of events is the creation of a signalling environment favouring allergy development, especially in those with a genetic predisposition. In this context, genetic predisposition is taken to indicate an existing bias towards the development of Th2 immunity and/or the presence of genetic polymorphisms in endogenous antipeptidases that could create supersensitivity to mite peptidases. Over the past 25 years, limited evidence that allergy may be associated with deficiencies in antipeptidase defence have accrued from functional studies (Eden *et al*., 2003; Hyde *et al*., 1979; Rudolph *et al*., 1978; Sigsgaard *et al*., 2000). More recently, these have been supplemented by genetic evidence, notably mutations in serpins (LETK-1, PAI-1, C1 esterase inhibitor, α_1-antichymotrypsin), which indicate an increased susceptibility to allergy in those with defective antipeptidase defences (Smith and Harper, 2006). Furthermore, evidence suggests that antipeptidase defences may be directly inactivated by certain allergens (Kalsheker *et al*., 1996; Brown *et al*., 2003). A key serpin present in epithelial lining fluid is α_1-antitrypsin which protects the respiratory tract from damage by serine peptidases that become activated during inflammation. The reactive centre of this protein is attacked proximal to the amino terminus by Der p 1, inactivating the inhibitor and thus potentiating any allergens with serine peptidase activity. Der p 1, and by implication related Group 1 mite allergens, appears to have an ability to evade inhibition by many mammalian antipeptidases. Of the mammalian antipeptidases examined, only α_2-macroglobulin inhibits Der p 1, and it is noteworthy that this antipeptidase is scarce in epithelial lining fluid (Hubbard and Crystal, 1991).

Several studies have documented the release of cytokines from airway epithelial cells by mite peptidase allergens and by peptidase-containing extracts or purified allergens derived from *A. fumigatus* or *P. chrysogenum* (Asokananthan *et al*., 2002b; King *et al*., 1998; Tai *et al*., 2006; Tomee *et al*., 1997, 1998; Sun *et al*., 2001). Although only a few examples from the gamut of peptidase allergens have been examined, major allergens from diverse sources converge on the same range of IgE-independent effector pathways. In airway epithelial cells, both Der p 1 and Der p 9 upregulate the transcription of cytokine genes and increase cytokine release (King *et al*., 1998; Sun *et al*., 2001). Similarly, peptidase activity has been associated with the production of PGE_2 and nitric oxide (Tai *et al*., 2006). These events involve, at least in part, signalling via pathways that are linked to cleavage of PARs. Der p 1, Der p 3 and Der p 9 all cleave the amino terminus of PAR-2 (Asokananthan *et al*., 2002b; Sun *et al*., 2001), the expression of which is upregulated in asthmatic airway epithelium (Knight

et al., 2001). These peptidases may cleave the receptor differentially, and the consequences may, therefore, depend upon the efficacy of the particular nascent N-terminal tethered ligands generated. Typically, cleavage of PAR-2 by trypsin yields an agonist N-terminus that increases phosphoinositide turnover, increases the intracellular concentration of calcium, and activates downstream MAPK signalling (Coughlin, 2000; Wang *et al*., 2002). As described later, the effects of mite peptidase allergens are less characterized, however.

Evidence therefore suggests that peptidase allergens liberate cytokines and chemokines that are relevant to allergy (Asokananthan *et al*., 2002b; Sun *et al*., 2001; Tai *et al*., 2006; Tomee *et al*., 1997, 1998). In epithelial cells stimulated by the serine peptidase mite allergens Der p 3 and Der p 6, this stimulation is accompanied by the release of eotaxin (CCL11), RANTES (CCL5) and GM-CSF (King *et al*., 1998; Sun *et al*., 2001). Other studies have documented the release of IL-6, IL-8 (CXCL8) and GM-CSF by Der p 1 (Asokananthan *et al*., 2002b). IL-6 has an essential role in B cell maturation and in IL-4-dependent IgE synthesis (Vercelli *et al*., 1989; Muraguchi *et al*., 1988), and it is known to be present in elevated amounts in bronchoalveolar lavage fluid or in nasal secretions in asthma (Broide *et al*., 1992; Fahy *et al*., 1995). GM-CSF generates signals that cause dendritic cells to differentiate and to migrate from the airway epithelium to present captured antigens at regional lymph nodes (Stick and Holt, 2003). IL-8 has a broad range of actions in addition to its chemoattractant properties, including promotion of plasma leakage and airway hyperresponsiveness (Colditz *et al*., 1990; De Sanctis *et al*., 1999; Fujimura *et al*., 1998; Xiu *et al*., 1995). It is present in elevated concentrations in asthma (Chanez *et al*., 1996; Nocker *et al*., 1996), especially prior to late phase reactions (Kurashima *et al*., 1996). RANTES is a chemokine for DCs (Sozzani *et al*., 1997), while together with eotaxin and GM-CSF, it is capable of upregulating eosinophil chemotaxis and activation (Rothenberg, 1999; Soloperto *et al*., 1991). A similar pattern of cytokine and chemokine release is evoked by peptidase allergens from *A. fumigatus* and *P. chrysogenum*, for which the release of PGE_2 and $TGF\beta_1$ has additionally been reported (Tai *et al*., 2006).

The effects of these signalling molecules may be further enhanced by the cleavage of receptor molecules in other cell types. Several targets have been identified. These include the low-affinity IgE receptor (CD23) on B-lymphocytes (Schulz *et al*., 1995) and α subunit of IL-2R (CD25) on T-lymphocytes (Schulz *et al*., 1998). Cleavage of the latter would diminish the tendency to Th1 immunity by suppressing IL-2 dependent T-cell proliferation. In DCs, potential targets are CD40 and the lectins DC-SIGN and DC-SIGNR, but the functional consequences remain to be established (Furmonaviciene *et al*., 2007).

The involvement of peptidase allergens in cleavage events at the epithelial surface or in other cells that have close associations with the airway mucosa raises the issue of what signal transduction processes are coupled to these events. Earlier reference was made to general mechanisms of peptidase signalling in epithelial cells, but the current understanding of signalling in the specific context of peptidase allergens is incomplete. Studies have been conducted with a miscellany of crude extracts, purified allergens, and with native or recombinant proteins which may be folded incorrectly or contaminated with traces of endotoxin. This diversity of trigger signal creates obvious uncertainties regarding interpretation of data, especially when this may be compounded by the use of epithelial cells of different lineage to provide the signal transduction readout. Generally, PAR activation is coupled *inter alia* to the turnover of phosphatidylinositol and an increase in intracellular calcium (Coughlin, 2000). Calcium responses have been observed for peptidase allergens, especially the mite serine peptidases (King *et al*., 1998; Sun *et al*., 2001), but it is less clear if the rise in calcium is

obligatory for the upregulation of cytokine expression through changes in gene transcription. The particular profile of cellular response is, thus, expected to depend on several factors, amongst which are the PAR sub-type, the agonist efficacy of the tethered ligand generated by proteolytic cleavage of the receptors and the particular G-proteins that are coupled to these receptors in any particular cell type. It remains possible that some cellular responses to peptidase allergens superficially appearing to be transduced by PAR-type mechanisms are mediated by entirely novel receptors, or through the agency of an intermediary peptidase liberated by an allergen. Of the HDM allergens, this possibility may be particularly relevant for Der p 1. Whether Der p 1 *per se* cleaves PAR-2 to elicit responses in epithelial cells is currently under debate.

PARs are archetypally responsive to serine peptidases, with PAR-2 being activated by trypsin and tryptase. In support of Der p 1 acting at least partially via PAR-2, epithelial cells exposed to Der p 1 release cytokines in a similar fashion to that which occurs on treatment with PAR-2 agonist peptide (Asokananthan *et al*., 2002a, 2000b). After activation, PARs are internalized and degraded by lysosomes. In epithelial cells previously stimulated with Der p 1, the Ca^{2+} response to trypsin or PAR-2 agonist peptide is diminished, consistent with Der p 1-PAR-2 cross reactivity (Asokananthan *et al*., 2002b). However, other evidence has suggested that Der p 1 may mediate at least some its peptidase-dependent actions through other mechanisms. In A549 cells, ERK1/2 signalling is the major MAPK signal transduction pathway for Der p 1 in the release of IL-8, whereas for Der p 3 and a PAR-2 agonist peptide the response additionally involved p38 and JNK signalling, and the amounts of IL-8 produced were greater (Adam *et al*., 2006). Although all three major MAPK pathways were activated by Der p 3, the responses differed temporally and in magnitude. Transcriptional regulation of IL-8 production by Der p 1 was dominated by NF-κB, whereas PAR-2-mediated responses of Der p 3 and PAR-2 agonist peptide involved both AP-1 and NF-κB (Adam *et al*., 2006). These differences do not prove the existence of a separate receptor for Der p 1, but they do highlight differences which may account for the lower potency of Der p 1 in the production of IL-8. Additional evidence for PAR-2-independent production of IL-8 comes from studies in KNRK cells transfected with the PAR-2 receptor. In these cells, Der p 1 was inactive in both increasing intracellular calcium and in stimulating the production of IL-8. However, trypsin or a PAR-2 agonist peptide achieved both (Adam *et al*., 2006). Moreover, in HeLa cells treated with Der p 1 a small increase in IL-8 production resulted without the cells having been transfected with the PAR-2 receptor (Adam *et al*., 2006). Whilst not proof that Der p 1 acts through a novel receptor, this too emphasizes that knowledge of these response pathways remains sketchy.

In contrast to the above, other transfection studies have suggested that Der p 1 does mediate at least some of its effects via PAR-2 activation (Asokananthan *et al*., 2002b). In HeLa cells transfected with enhanced yellow fluorescent protein constructs of either PAR-1 or PAR-2, Der p 1 caused receptor internalization and IL-6 release in only the cells transfected with the PAR-2 construct (Asokananthan *et al*., 2002b). This suggests that the cytokine release is associated with PAR-2 activation. Furthermore, in the A549 epithelial cell line which respond to PAR activation with an increase in intracellular calcium, Der p 1 pretreatment caused loss of responsiveness to PAR-2 agonist peptide and trypsin, but not PAR-1 agonist peptide and thrombin (Asokananthan *et al*., 2002b). This indicates that the calcium response is mediated by PAR-2 activation. However, these results are complex to understand because, when using cytokine production as a readout, pretreatment of A549 cells with Der p 1 had a different effect, in which cytokine release due to PAR-1 agonist peptide was diminished

whilst PAR-2 agonist peptide responses were retained (Asokananthan *et al*., 2002b). More recently, the fungal serine peptidase allergen Pen c13 has been shown to activate both PAR-1 and PAR-2, adding further complexity to understanding (Chui *et al*., 2007). One of the difficulties in understanding data from the A549 cell line is its possible heterogeneity, with at least four distinct morphological and antigenic sub-populations reported (Croce *et al*., 1999) and discrepancies in the expression of all four PARs.

BEAS-2B cells also respond to HDM peptidase allergens with an increase in intracellular calcium and the release of GM-CSF, IL-8 and IL-6. Here too, some evidence suggests that PAR-2 receptors do not mediate the calcium signalling response of Der p 1 (King *et al*., 1998). Calcium responses to Der p 9 were not affected by prior exposure to Der p 1, but were ablated by trypsin treatment (King *et al*., 1998). Although a commonly used surrogate of the airway epithelium, BEAS-2B cells are known to have a number of atypical features (Baker *et al*., 2003), so it is difficult to ascertain if the discrepant findings are an idiosyncrasy of this 'epithelial' cell line.

15.6 Conclusions

How allergens interact with the airway mucosa, and by implication with cells resident within the airway lumen, is a key step in the pathogenesis and maintenance of allergy. Mucosal surfaces encounter the largest amounts of inhaled allergens and it seems reasonable that much of allergy is dictated by events at these surfaces, and by the consequences of genetic predisposition. In addition to interactions between allergens and cells that bear surface-bound IgE, it has become recognized in recent years that allergens participate in interactions with the airway epithelium through mechanisms that are IgE-independent. These IgE-independent actions rely on properties of specific allergens that make them capable of catalysing certain types of reaction, or capable of activating receptors or other molecular recognition systems, to promote allergy development. It is perhaps surprising then that this aspect of allergen biology is still in its infancy. This status reflects a number of factors, not least the difficulties of obtaining pure allergens that retain all their biological properties. A further consideration is that the innate mechanisms with which allergens interact are themselves incompletely understood.

A pattern is emerging of how interactions with the airway epithelium deliver allergen and create a cytokine-rich milieu which promote allergy. However, one aspect that remains confusing is the cellular signalling which drives these events. Evidence surrounding the involvement of known PARs in responses to Group 1 mite allergens is contradictory, although a consensus exists that HDM serine peptidase allergens activate PAR-2. There is little doubt that MAPK signalling is central to these responses, but the finer details of which pathways are coupled to specific mediators will require further investigation.

In revisiting the question of what makes an allergen an allergen, it is clear that host tissue permeability is a factor which peptidase allergens are able to influence directly through their short-term actions on TJs. There is, therefore, no obligatory requirement for sensitization to inhaled allergens to depend upon a pre-existing or genetically programmed defect of mucosal permeability. Currently, evidence does not exist indicating a polymorphism for generally impaired TJ function in asthma, but there is a theoretical possibility of enhanced mucosal permeability if the repertoire of expressed TJ adhesion proteins were to become changed to a 'leakier' phenotype. Although the development of severe airway inflammation in asthma

does cause TJ disruption, more moderate disease does not appear to be associated with an unusually high permeability (Bennett and Ilowite, 1989; Ilowite *et al*., 1989), suggesting that in the lungs there are no major endogenous deficits in TJs or paracellular permeability control. This situation contrasts with skin, where accumulating evidence suggests two types of endogenous barrier defect predispose to allergy. In one of these, based on the strong association between atopy and Netherton's syndrome, the defect involves antipeptidase defence (Chavanas *et al*., 2000; Moffatt, 2004). In the other defect, it has been proposed that loss of function variants in filaggrin, a protein which is vital to aggregation of the keratin cytoskeleton and formation of the skin barrier, predisposes to atopic dermatitis. By extension, this defect may also increase susceptibility to other allergic conditions through other barriers where filaggrin is a significant component (Palmer *et al*., 2006; Weidinger *et al*., 2006). In principle, antipeptidase and structural barrier defects are not mutually exclusive and together create the possibility for enhancing the biochemical opportunism of major allergens.

A growing body of evidence suggests that enzymatic activity, especially peptidase activity, is important for sensitization to major allergens from fungi and arthropods. This emerging picture reveals new features of allergy, especially the co-dependence of acquired immunity on mechanisms of innate immunity which a number of allergens are able to activate through their broader biological properties. As discussed in this chapter, interesting implications arise from these insights into allergen biology. Particularly provocative is the suggestion that allergens could, regardless of source, be categorized into as few as two classes according to whether they required an adjuvant to elicit allergy. The other provocative insight is the opportunities for therapeutic exploitation created by the identification of upstream events in the allergy cascade which are not targeted by existing medications.

References

Aalberse RC (2000) Structural biology of allergens. *Journal of Allergy and Clinical Immunology*, **106**, 228–238.

Aalberse RC (2006) Structural features of allergenic molecules. *Chem.Immunol.Allergy*, **91**, 134–146.

Aalberse RC and Stadler BM (2006) In silico predictability of allergenicity: from amino acid sequence via 3-D structure to allergenicity. *Molecular and Nutritional Food Research*, **50**, 625–627.

Aas K (1978) What makes an allergen an allergen? *Allergy*, **33**, 3–14.

Adam E, Hansen KK, Astudillo FO, Coulon L, Bex F, Duhant X, Jaumotte E, Hollenberg MD, and Jacquet A (2006) The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *Journal of Biological Chemistry*, **281**, 6910–6923.

Aijaz S, Balda MS, and Matter K (2006) Tight junctions: molecular architecture and function. *International Review of Cytology*, **248**, 261–298.

Anderson JM and Van Itallie CM (1995) Tight junctions and the molecular basis for regulation of paracellular permeability. *American Journal of Physiology*, **269**, G467–G475.

Anderson JM, Balda MS, and Fanning AS (1993) The structure and regulation of tight junctions. *Current Opinion in Cell Biology*, **5**, 772–778.

Asokananthan N, Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS, Thompson PJ, and Stewart GA (2002a) Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *Journal of Immunology*, **168**, 3577–3585.

Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, and Stewart GA (2002b) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial

cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *Journal of Immunology*, **169**, 4572–4578.

Aurrand-Lions MA, Duncan L, Du PL, and Imhof BA (2000) Cloning of JAM-2 and JAM-3: an emerging junctional adhesion molecular family? *Current Topics in Microbiology and Immunology*, **251**, 91–98.

Bagarozzi DA, Jr. and Travis J (1998) Ragweed pollen proteolytic enzymes: possible roles in allergies and asthma. *Phytochemistry*, **47**, 593–598.

Bagarozzi DA, Jr., Pike R, Potempa J, and Travis J (1996) Purification and characterization of a novel endopeptidase in ragweed (*Ambrosia artemisiifolia*) pollen. *Journal of Biological Chemistry*, **271**, 26227–26232.

Bagarozzi DA, Jr., Potempa J, and Travis J (1998) Purification and characterization of an arginine-specific peptidase from ragweed (*Ambrosia artemisiifolia*) pollen. *American Journal of Respiratory Cell and Molecular Biology*, **18**, 363–369.

Baker SF, Yin Y, Runswick SK, Stewart GA, Thompson PJ, Garrod DR, and Robinson C (2003) Peptidase allergen Der p 1 initiates apoptosis of epithelial cells independently of tight junction proteolysis. *Molecular Membrane Biology*, **20**, 71–81.

Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, and Matter K (1996) Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *Journal of Cell Biology*, **134**, 1031–1049.

Balda MS, Flores-Maldonado C, Cereijido M, and Matter K (2000) Multiple domains of occludin are involved in the regulation of paracellular permeability. *Journal of Cellular Biochemistry*, **78**, 85–96.

Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, and Dejana E (2000) Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *Journal of Biological Chemistry*, **275**, 20520–20526.

Bennett WD and Ilowite JS (1989) Dual pathway clearance of ^{99m}Tc-DTPA from the bronchial mucosa. *American Review of Respiratory Disease*, **139**, 1132–1138.

Berger P, Tunon-de-Lara JM, Savineau JP, and Marthan R (2001) Selected contribution: tryptase-induced PAR-2-mediated $Ca^{(2+)}$ signaling in human airway smooth muscle cells. *Journal of Applied Physiol*ogy **91**, 995–1003.

Bojarski C, Weiske J, Schoneberg T, Schroder W, Mankertz J, Schulzke JD, Florian P, Fromm M, Tauber R, and Huber O (2004) The specific fates of tight junction proteins in apoptotic epithelial cells. *Journal of Cell Science*, **117**, 2097–2107.

Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, and Wasserman SI (1992) Cytokines in symptomatic asthma airways. *Journal of Allergy and Clinical Immunology*, **89**, 958–967.

Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C, Lamb J, and Sallenave JM (2003) House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *American Journal of Respiratory Cell and Molecular Biology*, **29**, 381–389.

Camerer E, Kataoka H, Kahn M, Lease K, and Coughlin SR (2002) Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells. *Journal of Biological Chemistry*, **277**, 16081–16087.

Chambers L, Brown A, Pritchard DI, Sreedharan S, Brocklehurst K, and Kalsheker NA (1998) Enzymatically active papain preferentially induces an allergic response in mice. *Biochemical and Biophysical Research Communications*, **253**, 837–840.

Chanez P, Enander I, Jones I, Godard P, and Bousquet J (1996) Interleukin 8 in bronchoalveolar lavage of asthmatic and chronic bronchitis patients. *International Archives of Allergy and Immunology*, **111**, 83–88.

Chavanas S, Bodemer C, Rochat A, Hamel-Teillac D, Ali M, Irvine AD, Bonafe JL, Wilkinson J, Taieb A, Barrandon Y, Harper JI, de Prost Y, and Hovnanian A (2000) Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. *Nature Genetics*, **25**, 141–142.

Chiu LL, Perng DW, Yu CH, Su SN, and Chow LP (2007) Mold allergen, pen C 13, induces IL-8 expression in human airway epithelial cells by activating protease-activated receptor 1 and 2. *Journal of Immunology*, **178**, 5237–5244.

Citi S (1993) The molecular organization of tight junctions. *Journal of Cell Biology*, **121**, 485–489.

Colditz IG, Zwahlen RD, and Baggiolini M (1990) Neutrophil accumulation and plasma leakage induced in vivo by neutrophil-activating peptide-1. *Journal of Leukocyte Biology*, **48**, 129–137.

Colloff MJ and Stewart GA (1997) House dust mites. In *Asthma*, Barnes PJ, Grunstein MM, Leff AR, and Woolcock AJ (eds), Lippincott-Raven, Philadelphia, PA, pp. 1089–1103.

Comoy EE, Pestel J, Duez C, Stewart GA, Vendeville C, Fournier C, Finkelman F, Capron A, and Thyphronitis G (1998) The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN-gamma. *Journal of Immunology*, **160**, 2456–2462.

Cortes L, Carvalho AL, Todo-Bom A, Faro C, Pires E, and Verissimo P (2006) Purification of a novel aminopeptidase from the pollen of *Parietaria judaica* that alters epithelial integrity and degrades neuropeptides. *Journal of Allergy and Clinical Immunology*, **118**, 878–884.

Coughlin SR (2000) Thrombin signalling and protease-activated receptors. *Nature*, **407**, 258–264.

Croce MV, Colussi AG, Price MR, Segal-Eiras A (1999). Identification and characterization of different subpopulations in a human lung adenocarcinoma cell line (A549). *Pathology and Oncology Research*, **5**, 197–204.

De Sanctis GT, MacLean JA, Qin S, Wolyniec WW, Grasemann H, Yandava CN, Jiao A, Noonan T, Stein-Streilein J, Green FH, and Drazen JM (1999) Interleukin-8 receptor modulates IgE production and B-cell expansion and trafficking in allergen-induced pulmonary inflammation. *Journal of Clinical Investigation*, **103**, 507–515.

Delventhal S, Brandis A, Ostertag H, and Pabst R (1992) Low incidence of bronchus-associated lymphoid tissue (BALT) in chronically inflamed human lungs. *Virchows Archive B Cell Pathology Including Molecular Pathology*, **62**, 271–274.

Dunnill MS (1960) The pathology of asthma, with special reference to changes in the bronchial mucosa. *Journal of Clinical Pathology*, **13**, 27–33.

Dunnill MS (1971) The pathology of asthma. *Ciba Foundation Study Group*, **38**, 35–46.

Ebnet K, Schulz CU, Meyer Zu Brickwedde MK, Pendl GG, and Vestweber D (2000) Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *Journal of Biological Chemistry*, **275**, 27979–27988.

Eden E, Hammel J, Rouhani FN, Brantly ML, Barker AF, Buist AS, Fallat RJ, Stoller JK, Crystal RG, and Turino GM (2003) Asthma features in severe alpha1-antitrypsin deficiency: experience of the National Heart, Lung, and Blood Institute Registry. *Chest*, **123**, 765–771.

Fahy JV, Wong H, Liu J, and Boushey HA (1995) Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *American Journal of Respiratory and Critical Care Medicine*, **152**, 53–58.

Fanning AS, Jameson BJ, Jesaitis LA, and Anderson JM (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *Journal of Biological Chemistry*, **273**, 29745–29753.

Fujimura M, Xiu Q, Tsujiura M, Tachibana H, Myou S, Matsuda T, and Matsushima K (1998) Role of leukotriene B4 in bronchial hyperresponsiveness induced by interleukin-8. *European Respiratory Journal*, **11**, 306–311.

Furmonaviciene R, Ghaemmaghami AM, Boyd SE, Jones NS, Bailey K, Willis AC, Sewell HF, Mitchell DA, and Shakib F (2007) The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clinical and Experimental Allergy*, **37**, 231–242.

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, and Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions [see comments]. *Journal of Cell Biology*, **123**, 1777–1788.

Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S, and Tsukita S (1994) Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *Journal of Cell Biology*, **127**, 1617–1626.

Furuse M, Sasaki H, Fujimoto K, and Tsukita S (1998) A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *Journal of Cell Biology*, **143**, 391–401.

Furuse M, Sasaki H, and Tsukita S (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. *Journal of Cell Biology*, **147**, 891–903.

Furuse M, Furuse K, Sasaki H, and Tsukita S (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *Journal of Cell Biology*, **153**, 263–272.

Garrod DR (1993) Desmosomes and hemidesmosomes. *Current Opinion in Cell Biology*, **5**, 30–40.

Gehr P and Heyder J (eds) (2000) *Particle–Lung Interactions*, Marcel Dekker, New York.

Ghaemmaghami AM, Gough L, Sewell HF, and Shakib F (2002) The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. *Clinical and Experimental Allergy*, **32**, 1468–1475.

Gough L, Schulz O, Sewell HF, and Shakib F (1999) The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *Journal of Experimental Medicine*, **190**, 1897–1902.

Gough L, Sewell HF, and Shakib F (2001) The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen. *Clinical and Experimental Allergy*, **31**, 1594–1598.

Gough L, Campbell E, Bayley D, Van Heeke G, and Shakib F (2003) Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model. *Clinical and Experimental Allergy*, **33**, 1159–1163.

Green KJ and Jones JC (1996) Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB Journal*, **10**, 871–881.

Grobe K, Becker WM, Schlaak M, and Petersen A (1999) Grass group I allergens (beta-expansins) are novel, papain-related proteinases. *European Journal of Biochemistry*, **263**, 33–40.

Grobe K, Poppelmann M, Becker WM, and Petersen A (2002) Properties of group I allergens from grass pollen and their relation to cathepsin B, a member of the C1 family of cysteine proteinases. *European Journal of Biochemistry*, **269**, 2083–2092.

Hassim Z, Maronese SE, and Kumar RK (1998) Injury to murine airway epithelial cells by pollen enzymes. *Thorax*, **53**, 368–371.

Herbert CA, Holgate ST, Robinson C, Thompson PJ, and Stewart GA (1990) Effect of mite allergen on permeability of bronchial mucosa [letter]. *Lancet*, **336**, 1132.

Herbert CA, King CM, Ring PC, Holgate ST, Stewart GA, Thompson PJ, and Robinson C (1995) Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *American Journal of Respiratory Cell and Molecular Biology*, **12**, 369–378.

Holt PG (2002) The role of airway dendritic cell populations in regulation of T-cell responses to inhaled antigens: atopic asthma as a paradigm. *Journal of Aerosol Medicine*, **15**, 161–168.

Holt PG, Schon-Hegrad MA, Oliver J, Holt BJ, and McMenamin PG (1990) A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *International Archives of Allergy and Applied Immunology*, **91**, 155–159.

Hou J, Gomes AS, Paul DL, and Goodenough DA (2006) Study of claudin function by RNA interference. *Journal of Biological Chemistry*, **281**, 36117–36123.

Hubbard RC and Crystal RG (1991) Antiproteases. In *The Lung: Scientific Foundation*, Crystal RG and West JB (eds), Raven Press, New York, pp. 1775–1787.

Huh JC, Strickland DH, Jahnsen FL, Turner DJ, Thomas JA, Napoli S, Tobagus I, Stumbles PA, Sly PD, and Holt PG (2003) Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC

activation occurs in the airway mucosa but not in the lung parenchyma. *Journal of Experimental Medicine*, **198**, 19–30.

Hyde JS, Werner P, Kumar CM, and Moore BS (1979) Protease inhibitor variants in children and young adults with chronic asthma. *Annals of Allergy*, **43**, 8–13.

Ilowite JS, Bennett WD, Sheetz MS, Groth ML, and Nierman DM (1989) Permeability of the bronchial mucosa to ^{99m}Tc-DTPA in asthma. *American Review of Respiratory Disease*, **139**, 1139–1143.

Jahnsen FL, Strickland DH, Thomas JA, Tobagus IT, Napoli, S, Zosky, GR, Turner DJ, Sly PD, Stumbles PA, Holt PG. (2006) Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus. *Journal of Immunology*, **177**, 5861–5867.

Kalb TH, Chuang MT, Marom Z, and Mayer L (1991) Evidence for accessory cell function by class II MHC antigen-expressing airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, **4**, 320–329.

Kalsheker NA, Deam S, Chambers L, Sreedharan S, Brocklehurst K, and Lomas DA (1996) The house dust mite allergen Der p1 catalytically inactivates alpha1- antitrypsin by specific reactive centre loop cleavage: a mechanism that promotes airway inflammation and asthma. *Biochemical and Biophysical Research Communications*, **221**, 59–61.

Kauffman HF, Tomee JF, Van De Riet MA, Timmerman AJ, and Borger P (2000) Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *Journal of Allergy and Clinical Immunology*, **105**, 1185–1193.

Kheradmand F, Kiss A, Xu J, Lee SH, Kolattukudy PE, and Corry DB (2002) A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *Journal of Immunology*, **169**, 5904–5911.

King C, Brennan S, Thompson PJ, and Stewart GA (1998) Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *Journal of Immunology*, **161**, 3645–3651.

Knight DA, Lim S, Scaffidi AK, Roche N, Chung KF, Stewart GA, and Thompson PJ (2001) Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. *Journal of Allergy and Clinical Immunology*, **108**, 797–803.

Kurashima K, Mukaida N, Fujimura M, Schroder JM, Matsuda T, and Matsushima K (1996) Increase of chemokine levels in sputum precedes exacerbation of acute asthma attacks. *Journal of Leukocyte Biology*, **59**, 313–316.

Kurup VP, Xia JQ, Shen HD, Rickaby DA, Henderson JD, Jr., Fink JN, Chou H, Kelly KJ, and Dawson CA (2002) Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp-f-2-induced immune response in mice. *International Archives of Allergy and Applied Immunology*, **129**, 129–137.

Lambrecht BN and Hammad H (2003) The other cells in asthma: dendritic cell and epithelial cell crosstalk. *Current Opinion in Pulmonary Medicine*, **9**, 34–41.

Machado DC, Horton D, Harrop R, Peachell PT, and Helm BA (1996) Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *European Journal of Immunology*, **26**, 2972–2980.

Mandell KJ and Parkos CA (2005) The JAM family of proteins. *Advanced Drug Delivery Reviews*, **57**, 857–867.

Matheson NR and Travis J (1998) Purification and characterization of a novel peptidase (IImes) from mesquite (*Prosopis velutina*) pollen. *Journal of Biological Chemistry*, **273**, 16771–16777.

Mezzetti M, Soloperto M, Fasoli A, and Mattoli S (1991) Human bronchial epithelial cells modulate CD3 and mitogen-induced DNA synthesis in T cells but function poorly as antigen-presenting cells compared to pulmonary macrophages. *Journal of Allergy and Clinical Immunology*, **87**, 930–938.

Miyoshi J and Takai Y (2005) Molecular perspective on tight-junction assembly and epithelial polarity. *Advanced Drug Delivery Reviews*, **57**, 815–855.

Moffatt MF (2004) SPINK5: a gene for atopic dermatitis and asthma. *Clinical and Experimental Allergy*, **34**, 325–327.

Mori L, Kleimberg J, Mancini C, Bellini A, Marini M, and Mattoli S (1995) Bronchial epithelial cells of atopic patients with asthma lack the ability to inactivate allergens. *Biochemical and Biophysical Research Communications*, **217**, 817–824.

Muraguchi A, Hirano T, Tang B, Matsuda T, Horii Y, Nakajima K, and Kishimoto T (1988) The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *Journal of Experimental Medicine*, **167**, 332–344.

Naylor B (1962) The shedding of the mucosa of the bronchial tree in asthma. *Thorax*, **17**, 69–72.

Nocker RE, Schoonbrood DF, van de Graaf EA, Hack CE, Lutter R, Jansen HM, and Out TA (1996) Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease. *International Archives of Allergy and Applied Immunology*, **109**, 183–191.

Pabst R and Gehrke I (1990) Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? *American Journal of Respiratory Cell and Molecular Biology*, **3**, 131–135.

Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, Goudie DR, Sandilands A, Campbell LE, Smith FJ, O'Regan GM, Watson RM, Cecil JE, Bale SJ, Compton JG, DiGiovanna JJ, Fleckman P, Lewis-Jones S, Arseculeratne G, Sergeant A, Munro CS, El Houate B, McElreavey K, Halkjaer LB, Bisgaard H, Mukhopadhyay S, and McLean WH (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature Genetics*, **38**, 441–446.

Piacentini GL, Vicentini L, Mazzi P, Chilosi M, Martinati L, and Boner AL (1998) Mite-antigen avoidance can reduce bronchial epithelial shedding in allergic asthmatic children. *Clinical and Experimental Allergy*, **28**, 561–567.

Platts-Mills TA (2007) The role of indoor allergens in chronic allergic disease. *Journal of Allergy and Clinical Immunology*, **119**, 297–302.

Radlowski M (2005) Proteolytic enzymes from generative organs of flowering plants (Angiospermae). *Journal of Applied Genetics*, **46**, 247–257.

Raftery MJ, Saldanha RG, Geczy CL, and Kumar RK (2003) Mass spectrometric analysis of electrophoretically separated allergens and proteases in grass pollen diffusates. *Respiratory Research*, **4**, 10.

Ritz SA, Gajewska BU, Stampfli MR, and Jordana M (2000) Determinants of the immune-inflammatory response in allergic airway inflammation: overview of antigen presentation and cellular activation. *Journal of Allergy and Clinical Immunology*, **106**, S206–S212.

Robinson C (1995) The airway epithelium: the origin and target of inflammatory airways disease and injury. In *Immunopharmacology of the Respiratory System*, Holgate ST (ed.), Academic Press, London, pp. 187–207.

Robinson C, Kalsheker NA, Srinivasan N, King CM, Garrod DR, Thompson PJ, and Stewart GA (1997) On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. *Clinical and Experimental Allergy*, **27**, 10–21.

Rothenberg ME (1999) Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *American Journal of Respiratory Cell and Molecular Biology*, **21**, 291–295.

Rudolph R, Dolling J, Kunkel G, Staud RD, and Baumgarten C (1978) The significance of nasal protease inhibitor concentrations in house dust allergy. *Allergy*, **33**, 310–315.

Salik E, Tyorkin M, Mohan S, George I, Becker K, Oei E, Kalb T, and Sperber K (1999) Antigen trafficking and accessory cell function in respiratory epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, **21**, 365–379.

Schechter NM, Brass LF, Lavker RM, and Jensen PJ (1998) Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *Journal of Cell Physiology*, **176**, 365–373.

Schulz O, Laing P, Sewell HF, and Shakib F (1995) Der p I, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *European Journal of Immunology*, **25**, 3191–3194.

Schulz O, Sewell HF, and Shakib F (1998) Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *Journal of Experimental Medicine*, **187**, 271–275.

Schulzke JD, Bojarski C, Zeissig S, Heller F, Gitter AH, and Fromm M (2006) Disrupted barrier function through epithelial cell apoptosis. *Annals of the New York Academy of Sciences*, **1072**, 288–299.

Sigsgaard T, Brandslund I, Omland O, Hjort C, Lund ED, Pedersen OF, and Miller MR (2000) S and Z alpha1-antitrypsin alleles are risk factors for bronchial hyperresponsiveness in young farmers: an example of gene/environment interaction. *European Respiratory Journal*, **16**, 50–55.

Smith PK and Harper JI (2006) Serine proteases, their inhibitors and allergy. *Allergy*, **61**, 1441–1447.

Soloperto M, Mattoso VL, Fasoli A, and Mattoli S (1991) A bronchial epithelial cell-derived factor in asthma that promotes eosinophil activation and survival as GM-CSF. *American Journal of Physiology*, **260**, L530–L538.

Sozzani S, Luini W, Borsatti A, Polentarutti N, Zhou D, Piemonti L, D'Amico G, Power CA, Wells TN, Gobbi M, Allavena P, and Mantovani A (1997) Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *Journal of Immunology*, **159**, 1993–2000.

Stewart GA (2000) The molecular biology of allergens. In *Asthma and Rhinitis*, Busse WW and Holgate ST (eds), Blackwell Science, Oxford, pp. 1107–1142.

Stewart GA and Robinson C (2003) Allergen Structure and Function. In *Middleton's Allergy. Principles and Practice*, Adkinson NF, Yunginger JW, Busse WW, Bochner BS, Holgate ST, and Simons FER (eds), Mosby, Philadelphia, PA, pp. 585–609.

Stick SM and Holt PG (2003) The airway epithelium as immune modulator: the LARC ascending. *American Journal of Respiratory Cell and Molecular Biology*, **28**, 641–644.

Sun G, Stacey MA, Schmidt M, Mori L, and Mattoli S (2001) Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *Journal of Immunology*, **167**, 1014–1021.

Tai HY, Tam MF, Chou H, Peng HJ, Su SN, Perng DW, and Shen HD (2006) Pen ch 13 allergen induces secretion of mediators and degradation of occludin protein of human lung epithelial cells. *Allergy*, **61**, 382–388.

Temkin V, Kantor B, Weg V, Hartman ML, and Levi-Schaffer F (2002) Tryptase activates the mitogen-activated protein kinase/activator protein-1 pathway in human peripheral blood eosinophils, causing cytokine production and release. *Journal of Immunology*, **169**, 2662–2669.

Tibbles LA and Woodgett JR (1999) The stress-activated protein kinase pathways. *Cellular and Molecular Life Science*, **55**, 1230–1254.

Tomee JF, Wierenga AT, Hiemstra PS, and Kauffman HK (1997) Proteases from *Aspergillus fumigatus* induce release of proinflammatory cytokines and cell detachment in airway epithelial cell lines. *Journal of Infectious Diseases*, **176**, 300–303.

Tomee JF, van Weissenbruch R, de Monchy JG, and Kauffman HF (1998) Interactions between inhalant allergen extracts and airway epithelial cells: effect on cytokine production and cell detachment. *Journal of Allergy and Clinical Immunology*, **102**, 75–85.

Tsukita S, Furuse M, and Itoh M (2001) Multifunctional strands in tight junctions. *Nature Reviews in Molecular Cell Biology*, **2**, 285–293.

Ubl JJ, Grishina ZV, Sukhomlin TK, Welte T, Sedehizade F, and Reiser G (2002) Human bronchial epithelial cells express PAR-2 with different sensitivity to thermolysin. *American Journal of Physiology–Lung Cell and Molecular Physiology*, **282**, L1339–L1348.

Van Itallie CM and Anderson JM (1997) Occludin confers adhesiveness when expressed in fibroblasts. *Journal of Cell Science*, **110**, 1113–1121.

van Rijt LS and Lambrecht BN (2005) Dendritic cells in asthma: a function beyond sensitization. *Clinical and Experimental Allergy*, **35**, 1125–1134.

Vercelli D, Jabara HH, Arai K, Yokota T, and Geha RS (1989) Endogenous interleukin 6 plays an obligatory role in interleukin 4-dependent human IgE synthesis. *European Journal of Immunology*, **19**, 1419–1424.

Vergnolle N, Wallace JL, Bunnett NW, and Hollenberg MD (2001) Protease-activated receptors in inflammation, neuronal signaling and pain. *Trends in Pharmacological Sciences*, **22**, 146–152.

Waetzig GH and Schreiber S (2003) Review article: mitogen-activated protein kinases in chronic intestinal inflammation – targeting ancient pathways to treat modern diseases. *Alimentary Pharmacology and Therapeutics*, **18**, 17–32.

Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, and Robinson C (1999) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *Journal of Clinical Investigation*, **104**, 123–133.

Wan H, Winton HL, Soeller C, Gruenert DC, Thompson PJ, Cannell MB, Stewart GA, Garrod DR, and Robinson C (2000a) Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clinical and Experimental Allergy*, **30**, 685–698.

Wan H, Winton HL, Soeller C, Stewart GA, Thompson PJ, Gruenert DC, Cannell MB, Garrod DR, and Robinson C (2000b) Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBEo$^-$. *European Respiratory Journal*, **15**, 1058–1068.

Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, Cannell MB, Stewart GA, Garrod DR, and Robinson C (2001) The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clinical and Experimental Allergy*, **31**, 279–294.

Wang H, Ubl JJ, Stricker R, and Reiser G (2002) Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. *American Journal of Physiology – Lung Cell and Molecular Physiology*, **283**, C1351–C1364.

Weidinger S, Illig T, Baurecht H, Irvine AD, Rodriguez E, Diaz-Lacava A, Klopp N, Wagenpfeil S, Zhao Y, Liao H, Lee SP, Palmer CN, Jenneck C, Maintz L, Hagemann T, Behrendt H, Ring J, Nothen MM, McLean WH, and Novak N (2006) Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *Journal of Allergy and Clinical Immunology*, **118**, 214–219.

Wong V and Gumbiner BM (1997) A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *Journal of Cell Biology*, **136**, 399–409.

Xiu Q, Fujimura M, Nomura M, Saito M, Matsuda T, Akao N, Kondo K, and Matsushima K (1995) Bronchial hyperresponsiveness and airway neutrophil accumulation induced by interleukin-8 and the effect of the thromboxane A_2 antagonist S-1452 in guinea-pigs. *Clinical and Experimental Allergy*, **25**, 51–59.

16

The Epithelium as a Regulator of Airway Inflammation

Richard Leigh and **David Proud**
Departments of Medicine and Physiology & Biophysics, Institute of Infection, Immunity and Inflammation, University of Calgary, Alberta, Canada

16.1 Introduction

The airway epithelium is in a unique position at the interface between the host and the external environment, and is constantly exposed to a wide range of exogenous and endogenous stimuli. Although classically considered a barrier to inhaled noxious gases, particulates, bacteria, viruses, allergens, and other substances in inspired air, there is now substantial evidence that epithelial cells play a critical role in regulating immune-mediated airway inflammation. In response to a variety of exogenous and endogenous stimuli, airway epithelial cells become functionally active to produce and release a wide array of biologically active compounds, including cytokines, growth factors, chemokines, lipid-mediators, peptides, proteases and reactive oxygen species. These mediators can have important roles, not only in normal physiological processes, but also in the initiation and progression of various airway inflammatory disorders, such as asthma, smoking-related chronic bronchitis, acute infective bronchitis, bronchiectasis and cystic fibrosis. The epithelium can also contribute, however, to the regulation of over-exuberant inflammation via its ability to inhibit or catabolize a range of inflammatory mediators. This chapter describes our current understanding of the critical role that epithelial cells play in regulating inflammatory and repair processes in the airway both by the production of pro-inflammatory mediators and via their catabolic and inhibitory functions.

16.2 Epithelial production of cytokines, growth factors and chemokines

16.2.1 Cytokines and growth factors

The airway epithelium is a major contributor to the local cytokine network in the airway. Epithelial cells can respond to numerous stimuli to produce and release a wide array of

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

cytokines, chemokines, colony-stimulating factors and growth factors, as well as several cytokine antagonists. They can also show increased expression of adhesion molecules for inflammatory cells. All of these responses can regulate the inflammatory status of the airway, and contribute to the pathogenesis of inflammatory airway disorders.

Cytokines are extra-cellular signalling proteins, usually less than 80 kDa in size, that are involved in cell-to-cell interactions through specific receptors on the surface of target cells. They usually have an effect on closely adjacent cells and therefore function in a predominantly paracrine fashion, although they may also act in an endocrine or autocrine manner. They act on target cells to cause a wide array of cellular functions including activation, proliferation, chemotaxis, immunomodulation, release of other cytokines or mediators, cell growth and differentiation, and apoptosis (Chung and Barnes, 1999). Airway epithelial cell derived cytokines may, therefore, amplify ongoing inflammatory processes via the recruitment and activation of specific subsets of inflammatory cells, as well as by enhancing their survival in the airway microenvironment. In addition, airway epithelial cells can initiate inflammatory cascades by generating cytokines in direct response to viral and bacterial products, noxious gases, and sensitizing chemicals. Airway epithelial cells also represent targets for cytokines that act in a paracrine fashion, which may then modulate airway epithelial cell functions.

Epithelial cells are capable of releasing numerous multifunctional cytokines, growth factors and colony-stimulating factors that can exert effects on multiple cell targets (Figure 16.1). Among the pleiotropic cytokines produced by epithelial cells, interleukin (IL)-6 and IL-11 have been studied in most detail. Although cultured epithelial cells constitutively release IL-6 (Cromwell *et al*., 1992, Subauste *et al*., 1995), production is greatly increased by a variety of stimuli, including histamine, IL-1β and TNFα (Cromwell *et al*., 1992), viral infection (Subauste *et al*., 1995), and both fungal and dust mite proteases (King *et al*., 1998). In vivo, enhanced epithelial gene expression of IL-6 is seen in bronchial biopsies from asthmatic subjects, and concentrations of IL-6 are increased in bronchoalveolar lavage (BAL) fluid from symptomatic asthmatic subjects (Mattoli *et al*., 1991). Epithelial production of IL-6 may modulate allergic diseases because it is known to induce B-cell differentiation, T-cell proliferation and activation, neural differentiation, and enhanced mucosal IgA production (Kishimoto *et al*., 1992). Transgenic mice that overexpress IL-6 have pronounced peribronchial infiltration of lymphocytes but, surprisingly, have reduced airway responsiveness (AHR) to methacholine (DiCosmo *et al*., 1994). IL-11 is a cytokine with similarities to IL-6. Epithelial expression of IL-11 is increased in asthma and during respiratory viral infections (Einarsson *et al*., 1996). Although the role of IL-11 in the pathogenesis of airway diseases is not fully understood, it is known that IL-11 can activate B cells by means of a mechanism that is T-cell dependent, and bronchial challenge of mice with IL-11 leads to pronounced AHR and mononuclear cell infiltration (Einarsson *et al*., 1996). Furthermore, targeted overexpression of IL-11 in the airway of adult mice induces airway remodelling characterized by subepithelial fibrosis (Ray *et al*., 1997).

Epithelial cells also produce thymic stromal lymphopoietin (TSLP), a cytokine that has attracted particular attention because of its role in the regulation of dendritic cell function (see Chapter 11). TSLP is an IL-7-like cytokine produced mainly by barrier epithelial cells, and TSLP expression is increased in the airway of asthmatic subjects and correlates with symptom severity (Ying *et al*., 2005). TSLP has been reported to prime dendritic cells to promote Th2 cell differentiation and may thus play a role in sensitization toward allergens (Huston and Liu, 2006; Soumelis *et al*., 2002).

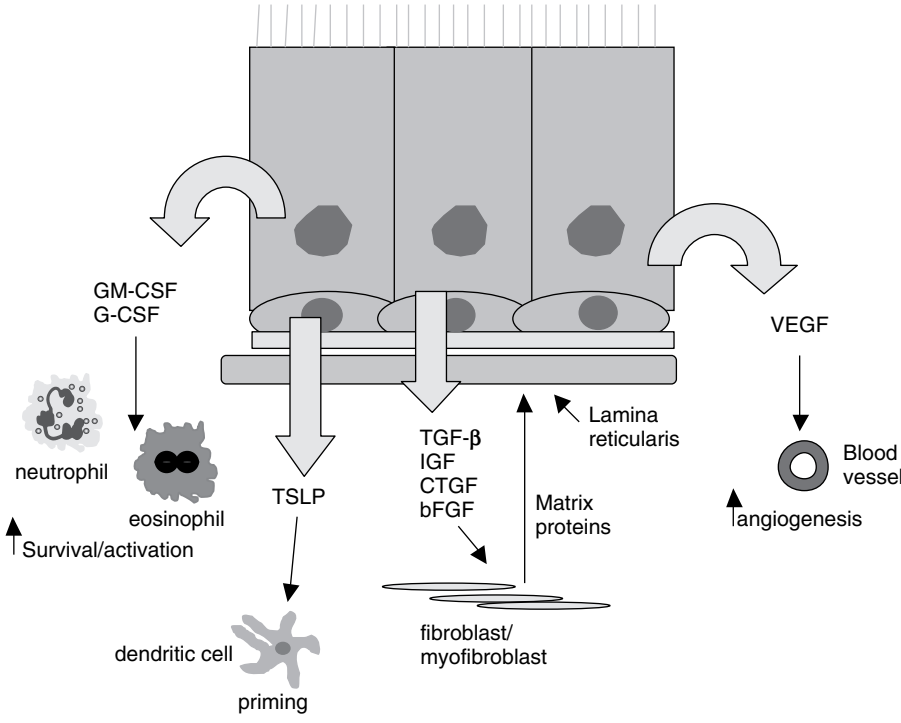


Figure 16.1 Cytokines and growth factors released by epithelial cells can affect multiple cell types within the airway. Growth factors such as GM-CSF and G-CSF can enhance the survival and activation of leukocyte populations, while production of thymic stromal lymphopoietin (TSLP) can prime dendritic cells to polarize T-cells to a Th2 phonotype. Vascular endothelial growth factor (VEGF), as well as several chemokines and peptides can enhance angiogenesis. The epithelium also releases a number of growth factors, including TGF-β, that can enhance the proliferation of fibroblasts and differentiation to myofibroblasts, such that they will release matrix proteins that can contribute to thickening of the lamina reticularis

The likely role in airway disease of other pleiotropic cytokines produced from epithelial cells is more difficult to predict. Although low levels of IL-1β are secreted, large amounts of IL-1β are seen when cells are exposed to cytotoxic stimuli, or are lysed (Kenney *et al*., 1994). Epithelial cells also contain large quantities of intracellular IL-1 receptor antagonist type I (icIL-1ra), however, so the role of epithelial IL-1β may vary depending on relative production to that of IL-1ra (Yoon *et al*., 1999). Epithelial cells obtained from bronchial brushings from healthy subjects produce the immunoregulatory cytokine IL-10, whereas production is greatly reduced in cells from patients with cystic fibrosis (Bonfield *et al*., 1995). Epithelial cells also produce IL-15 and low levels of TNFα.

The production of interferons by airway epithelial cells has recently attracted considerable interest, particularly in the context of viral infections. Although it is generally agreed that epithelial cells do not produce the prototypical type II interferon, IFN-γ, there is controversy regarding type I interferons. Although mRNA expression of IFN-α and IFN-β has consistently been detected, results of protein release have been more variable, perhaps due to varying stimuli and the relative limitations of current ELISAs. While some investigators

have failed to detect type I IFNs from cultured epithelial cells infected with rhinovirus (Spurrell *et al*., 2005), it has recently been suggested that impaired epithelial production of IFN-β in response to rhinovirus infection may contribute to viral exacerbations of asthma (Wark *et al*., 2005). A recent study also suggests that deficient type III, IFN-λ1 (IL-29) and IFN-λ2/3 (IL-28A/B) production may contribute to asthma exacerbations (Contoli *et al*., 2006). Further studies are needed to confirm these important observations.

Increased granulocyte-macrophage colony-stimulating factor (GM-CSF) expression has also been observed in epithelial cells from individuals with symptomatic allergic rhinitis (Nonaka *et al*., 1996), and from bronchial biopsies of subjects with asthma (Marini *et al*., 1992). Moreover, levels of GM-CSF expression correlate with the extent of eosinophil infiltration of the airway epithelium (Wang *et al*., 1994). GM-CSF could modulate allergic inflammation via its abilities to prolong eosinophil survival and to activate eosinophils, neutrophils and macrophages to display enhanced cytotoxic activity, generation of mediators and phagocytosis. Granulocyte colony-stimulating factor (G-CSF) is also produced by airway epithelial cells and can enhance the survival and activation of neutrophils.

The epithelium is a significant source of a number of growth factors that can act on several cell types within the airway (Figure 16.1). Airway epithelial cells express several members of the epidermal growth factor (EGF) family, including EGF, heparin-binding epidermal growth factor (HB-EGF), isoforms of transforming growth factor (TGF)-β, activin A and amphiregulin (Knight and Holgate, 2003). The epithelium also expresses three members of the epithelial growth factor receptor family (EGFR) (c-erbB1, c-erbB2 and c-erbB3), and when epithelial surfaces are injured, the normal response is for epithelial cells to upregulate members of the EGFR family. Activation of the EGF receptor leads to mucin gene expression and epithelial proliferation, differentiation and repair. Interestingly, epithelial expression of both EGF and EGFR is increased in asthma (Polosa *et al*., 2002). In culture models, epithelial cells constitutively produce vascular endothelial growth factor (VEGF) and this is enhanced in response to respiratory viral infections (Psarras *et al*., 2006). VEGF production could contribute to angiogenesis and increased vascular permeability in asthma. Stem cell factor (SCF) is also produced by the epithelium, and levels of SCF correlate with numbers of epithelial mast cells in the upper airway (Otsuka *et al*., 1998).

Epithelial cells in the process of repair, or in response to other stimuli, elaborate a variety of growth factors that enhance proliferation of fibroblasts and differentiation of these cells into activated myofibroblasts. These include several isoforms of transforming growth factor (TGF)β, connective tissue growth factor (CTGF), insulin-like growth factors (IGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). It has been reported that TGFβ1, but not other isoforms, enhances the speed of epithelial wound repair. Interestingly, in a murine model of allergen-induced airway remodelling, the airway epithelium was found to be the main source of TGFβ1 within the airway wall (Kelly *et al*., 2005). Epithelial production of a number of growth factors also has been shown to be upregulated in response to mechanical strain (Tschumperlin and Drazen, 2006).

16.2.2 Chemokines

Chemokines are a large superfamily of small (approximately 8–15 kDa), structurally related cytokines with potent leukocyte activation and/or chemotactic activity. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines. Thus far, approximately 50 chemokines have been identified, along

with some 20 or so receptors (Allen *et al*., 2007). Chemokines have been characterized into four subgroups (Colobran *et al*., 2007). The majority of chemokines are classified as members of either the α, or CXC, chemokine family, or the β, or CC, chemokine family. Both of these subfamilies contain four cysteine residues in conserved locations that are critical to forming their three-dimensional shape. The classification into subfamilies is based on whether the two amino terminal cysteine residues are immediately adjacent, or separated by one amino acid. The CXC chemokines contain a single amino acid between the first and second cysteine residues, while the CC chemokines have adjacent cysteine residues. The CXC family is further subdivided into two classes, based on the presence or absence of the amino-terminal sequence Glu-Leu-Arg (ELR). The ELR-containing CXC chemokines are predominantly chemoattractant for neutrophils, while the non-ELR subgroup is chemoattractant for lymphocytes and other cells. By contrast, CC chemokines generally attract monocytes, lymphocytes, basophils, and eosinophils. Two smaller subfamilies of chemokines also exist. The C group is derived from only two highly homologous genes encoding XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β). These two chemokines differ by only two amino acid residues and both bind to the XCR1 receptor. Lymphokines lack two of the four-cysteine residues and, thus have only one disulfide bond, but they share homology with a number of CC chemokines. XCL1 and 2 are potent chemoattractants for T and NK cells, but not for monocytes or neutrophils. Fractalkine (CX3CL1) is the only member of the CX3C chemokine family, and has three amino acid residues between the first two cysteine residues. By contrast to most chemokines, it tends to be tethered to the extracellular surface of cells that produce it (Colobran *et al*., 2007). The epithelium can produce a broad array of chemokines of the CXC, CC and CX3C classes. As such, epithelial cells can regulate the recruitment of a wide variety of inflammatory cell types into the airway, depending on the stimuli to which they are exposed, the profile of chemokines produced, and the extent to which selected inflammatory cell populations are primed to respond (Figure 16.2).

Airway epithelial cells produce both classes of CXC chemokines. The prototypical ELR-containing CXC chemokine produced in large quantities by epithelial cells is IL-8 (CXCL8), a potent neutrophil chemoattractant. In vivo, epithelial cell expression of IL-8 is increased in allergic rhinitis and asthma. Epithelial expression of IL-8 is induced in response to a wide variety of stimuli, including cytokines, pollutants, allergens, and mechanical strain. Given the prominent neutrophilic response following exposure of the airway to infectious agents, it is of interest that inhaled pathogens, particularly respiratory viruses, are potent inducers not only of IL-8 but also of ENA-78 (CXCL5), Gro-α (CXCL1) and Gro-γ (CXCL3). Epithelial cells also make large amounts of the non-ELR CXC chemokines, monokine induced by γ-IFN (Mig, CXCL9), IFN-inducible protein of 10 kDa (IP-10, CXCL10) and IFN-inducible T-cell α-chemoattractant (I-TAC, CXCL11). All of these chemokines are ligands for the CXCR3 receptor found predominantly on Type 1 (Th1) lymphocytes and on NK cells. As their names imply, all of these chemokines are induced by type I and type II interferons. Infection of epithelial cells with respiratory viruses can also induce CXC chemokines. Interestingly, while infection of epithelial cells with human rhinovirus induces production of CXCL10, this occurs independently of interferon induction (Spurrell *et al*., 2005). Epithelial generation of most CXC chemokines is not particularly sensitive to inhibition by glucocorticoids.

Epithelial cells also produce many members of the CC chemokine family that can function as chemoattractants for eosinophils, basophils, monocytes, dendritic cells and lymphocytes, depending upon their specific receptor usage. Eotaxin (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26) are selective ligands for the CCR3 chemokine receptor. Gene expression

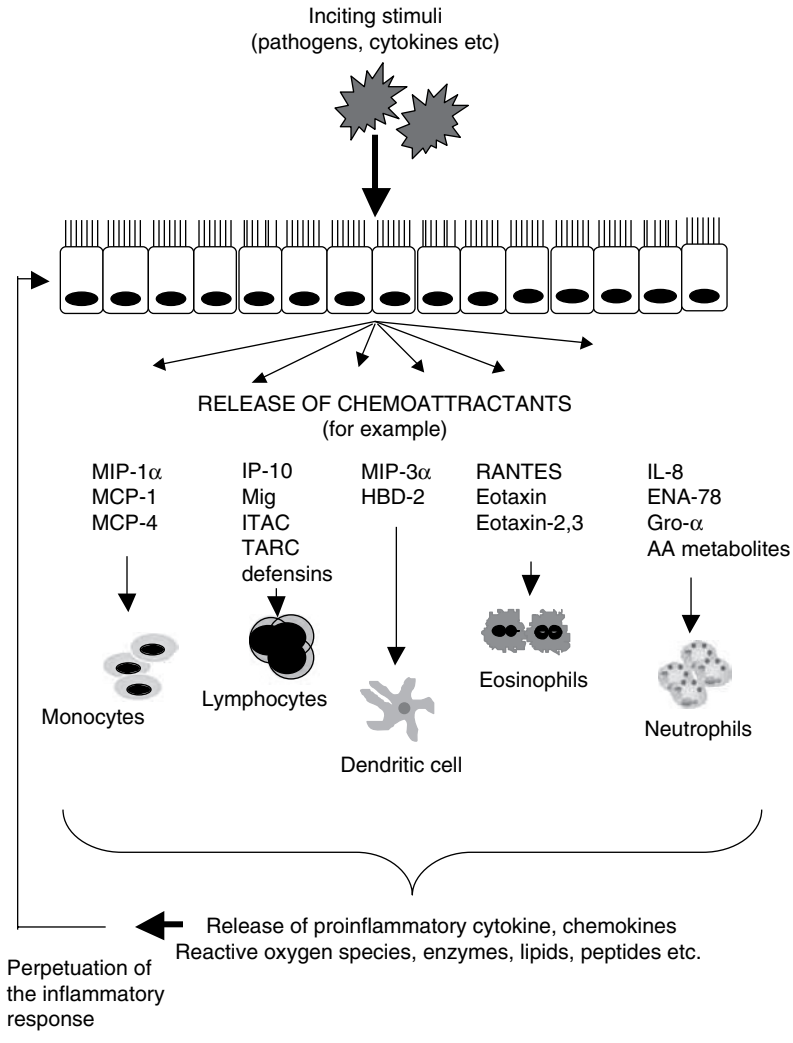


Figure 16.2 Epithelial cells can release chemoattractants for multiple types of inflammatory cells. Depending upon the specific inciting stimulus, epithelial cells can release chemokines and other chemoattractants (see text for abbreviations) that can recruit and activate neutrophils, eosinophils, dendritic cells, monocytes and lymphocytes. Depending upon the spectrum of cells recruited and activated, a range of pro-inflammatory products will be released. Several of these will feed back to further activate the epithelium causing a perpetuation of the inflammatory response

of CCL11, CCL24 and CCL26 is increased in the airway epithelium of asthmatic individuals and can be induced in vitro by exposure of epithelial cells to either IL-4 or IL-13 (van Wetering *et al*., 2007). The predominant eosinophil chemoattractant released by epithelial cells, however, is RANTES (regulated on activation, normal T-cell expressed and secreted, CCL5). CLL5 levels are increased in the BAL fluids of asthmatic compared with normal subjects, and eosinophil recruitment after allergen challenge is associated with increased

levels of CCL5 in BAL fluid. Indeed, CCL5, together with IL-5, is reported to be the major eosinophil chemoattractant in the asthmatic airway (Venge *et al*., 1996). Increased expression of CCL5 also is detected in the epithelium of nasal polyps, in the airway secretions of subjects during virally induced asthma exacerbations, and in secretions from allergic subjects after allergen challenge. The airway epithelium also produces mucosal-associated epithelial chemokine (MEC, CCL28), macrophage inflammatory protein (MIP)-1α (CCL3), and monocyte chemoattractant protein (MCP)-1 (CCL2). MCP-4 (CCL13) can be induced by inflammatory stimuli in vitro, and is upregulated in the airway epithelium of asthmatic patients, as well as in patients with sinusitis. Of particular interest is the ability of epithelial cells to produce substantial amounts of MIP-3α (CCL20) in response to a variety of stimuli, including ambient particulates and several cytokines (Reibman *et al*., 2003). CCL20 is a ligand for the CCR6 receptor found on immature dendritic cells, and its chemoattractant properties for these cells could play an important role in linking innate and adaptive immunity.

More recently, airway epithelial cells have been shown to produce thymus and activation-regulated chemokine (TARC, CCL17) in response to several stimuli, including whole allergen extract of house dust mite (Heijink *et al*., 2007). TARC may be critical in Th2 cell recruitment in allergic airway inflammation. Not only are increased levels of TARC found in BAL after allergen challenge of allergic asthmatic subjects (Bochner *et al*., 2003), but increased epithelial expression of TARC is also seen (Panina-Bordignon *et al*., 2001). The majority of Th2 cells express CCR4 (Kim *et al*., 2001), the receptor ligand for TARC protein, and an allergen-induced increase in $CCR4^+$ T cells has been observed in asthmatic airway (Panina-Bordignon *et al*., 2001).

Fractalkine (CX3CL1) is the only member of the CX3C chemokine family. The specific receptor for fractalkine, CX3CR1, is expressed on monocytes, T-lymphocytes, mast cells and NK cells. IFNγ stimulates epithelial expression of fractalkine, although the majority of fractalkine remains membrane tethered (Fujimoto *et al*., 2001). A recent study performed in atopic asthmatic patients has reported that segmental allergen challenge resulted in significant upregulation of BAL fractalkine, and that immunohistochemistry staining before and 24 hours after allergen provocation confirmed airway epithelial staining of fractalkine (Rimaniol *et al*., 2003). Interestingly, not only was adhesion of mononuclear cells to IFNγ stimulated epithelial cells in vitro partially inhibited by antibodies to fractalkine, but levels of fractalkine in BAL fluids from subjects with inflammatory airway diseases correlate with mononuclear cell counts in the fluids (Fujimoto *et al*., 2001).

Interestingly, recent studies have demonstrated the presence of several functional chemokine receptors, including CCR3, CXCR3, and CXCR4 on airway epithelial cells (Beck *et al*., 2006; Kelsen *et al*., 2004; Eddleston *et al*., 2002), raising the possibility that epithelial chemokine release also may act in an autocrine fashion.

16.3 Epithelial production of lipid mediators

Epithelial cells have the ability to convert arachidonic acid to a range of biologically active metabolites. These mediators are expressed both constitutively, and in response to a variety of stimuli. Once free arachidonic acid is released from membrane phospholipid stores, it is subject in epithelial cells to oxidation along one of three major metabolic pathways, (1) the lipoxygenase pathway, which produces the leukotrienes, mid-chain hydroxyeicosatetraenoic acids (HETEs) and lipoxins; (2) the cyclooxygenase (COX) pathway, which produces the

prostaglandins, and (3) the cytochrome P-450 monoxygenase pathway, which produces midchain and ω-terminal HETEs as well as cis-epoxyeicosatrienoic acids (EETs).

Although epithelial cells from all mammals examined metabolize arachidonic acid via lipoxygenase pathways, considerable species variations exist in terms of the dominant pathway used. Thus, while 5-lipoxygenase activity is evident in canine and ovine epithelial cells, metabolism via 15-lipoxygenase predominates in human airway epithelial cells. The 15-lipoxygenase pathway converts arachidonic acid to 15-HETE, as well as a variety of biologically active hydroperoxy, epoxyhydroxy, keto, and dihydroxy acids (Holtzman, 1992). Expression of 15-lipoxygenase is increased in the epithelium of asthmatic individuals, and elevated levels of 15-HETE are found in the BAL fluid of asthmatic subjects after allergen challenge, where it is thought to potentiate 5-lipoxygenase activity in leukocytes and enhance the early bronchoconstrictor response to inhaled allergen (Kumlin *et al*., 1990). Once produced, 15-HETE can enhance epithelial mucus glycoprotein generation and augment the acute response to allergen challenge in asthmatics (Lai *et al*., 1990). Another metabolite, 8S, 15S-diHETE, induces neutrophil chemotaxis. The recruited neutrophils can then utilize 15-HETE to generate the trihydroxy acid lipoxin A by a transcellular pathway catalysed by 5-lipoxygenase. Lipoxin A inhibits the cytotoxic activity of human natural killer (NK) cells, causes superoxide generation by neutrophils, and contracts human bronchi.

Human bronchial epithelial cells express both the COX-1 and COX-2 isoforms of cyclooxygenase under basal conditions, but the expression of COX-2 is further enhanced in vitro by inflammatory stimuli, such as IL-1β and bradykinin (Petkova *et al*., 1999). Moreover, epithelial expression of COX-2 is markedly enhanced in asthmatic individuals (Redington *et al*., 2001). The dominant COX pathway products generated in human epithelial cells are Prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ (Churchill *et al*., 1989), and production of both products is enhanced in response to several pro-inflammatory stimuli, including bradykinin and histamine. Increased levels of both prostanoids also are detected in BAL fluids of asthmatic and atopic subjects, and levels increase further in response to allergen provocation (Liu *et al*., 1991). In vitro, PGE_2 has several anti-inflammatory actions. It inhibits mast cell degranulation and production of LTB4 by alveolar macrophages, and also mediates relaxation of airway smooth muscle and regulates mucus glycoprotein secretion. In vivo, inhalation of PGE_2 blocks early and late bronchoconstrictor responses to inhaled allergen and abolishes allergen-induced increase in bronchial reactivity (Pavord *et al*., 1993). In addition, it attenuates exercise-induced bronchoconstriction (Melillo *et al*., 1994). On the other hand, PGE_2 increases cough sensitivity and may play a role in the cough associated with angiotensin-converting enzyme-inhibitor therapy. By contrast, $PGF_{2\alpha}$ is a potent bronchoconstrictor mediator (Holtzman, 1992).

Airway epithelial cells also express cytochrome P-450 monoxygenase enzymes with the capacity to metabolize arachidonic acid into a series of regiospecific and stereospecific fatty acid epoxides and alcohols. These include midchain and ω-terminal HETEs as well as cis-epoxyeicosatrienoic acids (EETs). Although most P-450 enzymes are primarily expressed in the liver, the CYP2J isoform of P-450 is present in both ciliated and non-ciliated human airway epithelial cells, and metabolites of the P-450 pathway affect epithelial ion transport as well as bronchomotor tone (Jacobs and Zeldin, 2001).

Finally, it should be noted that human bronchial epithelial cells also synthesize platelet activating factor (PAF), although the majority remains cell associated (Holtzman *et al*., 1991). Interestingly, human airway epithelial cells also have functional receptors for PAF (Kang *et al*., 1994), stimulation of which, in the presence of arachidonic acid, can induce production of 15-HETE (Salari and Schellenberg, 1991).

16.4 Epithelial production of peptide mediators

Airway epithelial cells generate a variety of peptide mediators, including human β-defensin (HBD)-1–4 and the cathelicidin, LL-37. HBD-1 is constitutively expressed and production of this molecule is modulated little by inflammatory stimuli. By contrast, HBD-2, 3, and 4 can be induced by a variety of stimuli, including cytokines, such as IL-17, and viral infection. Interestingly, synergistic induction of HBD-2 is observed when cells are infected with rhinovirus in the presence of IL-17. Cathelicidin is also constitutively produced, but expression, again, can be enhanced by inflammatory stimuli. In addition to their roles in host defence (see Chapter 10), these peptides can contribute to the regulation of inflammation. Human β-defensins are chemotactic for immature dendritic cells and some types of T cells (Yang *et al*., 2004), and so could enhance recruitment of these cell types to the airway. LL-37 is chemotactic for neutrophils, eosinophils and monocytes, and can stimulate angiogenesis via actions mediated by interactions with the formyl peptide receptor-like 1 (Tjabringa *et al*., 2005).

Epithelial cells from various animal species have been reported to produce peptides including vasopressin, substance P and calcitonin gene-related peptide (CGRP), but there is little data on production of these peptides by human cells. Human airway epithelial cells do, however, produce endothelin-1 (ET-1) and endothelin-3 (ET-3), which are potent bronchoconstrictor peptides. Cultured epithelial cells produce low levels of ET-1 at baseline, but several stimuli, including infection with respiratory syncytial virus (Behera *et al*., 1998), exposure to thrombin, or any of several cytokines, have been shown to enhance ET-1 synthesis and release in vitro. By contrast, production is inhibited upon treatment with glucocorticoids, IFNγ, or PDGF. In vivo, expression of the gene for preproendothelin, and production of endothelin-1 peptide, is increased in epithelial cells from asthmatic subjects (Ackerman *et al*., 1995). Elevated levels of endothelins are also found in BAL fluids from asthmatic subjects, and BAL endothelin levels correlate with asthma severity (Nomura *et al*., 1989). Intranasal administration of endothelin-1 induces sneezing and increased nasal secretions, but does not induce increased vascular permeability (Riccio *et al*., 1995).

Once generated, endothelins not only cause bronchoconstriction, but stimulate mucus production and airway microvascular leakage (Goldie and Henry, 1999). They can also serve as autocrine regulators capable of stimulating epithelial production of cyclooxygenase and lipoxygenase lipid mediators (Markewitz *et al*., 1995). Endothelins can induce smooth muscle proliferation and promote airway remodelling (Goldie and Henry, 1999). Moreover, there is evidence that endothelin receptor stimulation can potentiate cholinergic nerve-mediated contraction in human airway (Fernandes *et al*., 1996), supporting the concept that endothelins may have a mediator role in bronchial obstruction in airway diseases.

16.5 Epithelial production of reactive nitrogen and oxygen species

Although reactive oxygen species (ROS) and reactive nitrogen species (RNS) have traditionally been thought of as being produced primarily by macrophages, and leukocytes, airway epithelial cells are increasingly recognized as being an important cellular source of ROS and RNS. Reactive oxygen species can be formed by a variety of enzymes, including xanthine

oxidases, cyclooxygenase, NADPH oxidase and the dual oxidases (Duox) 1 and 2, which have both been detected in airway epithelium (Geiszt *et al*., 2003). It has been shown that airway epithelial cells from several species, including humans, release hydrogen peroxide at baseline (Figure 16.3(A)). Although the amount produced is less than from macrophages, when taken as a whole, the epithelial surface lining the entire respiratory tract could be a significant source of reactive oxygen molecules (Kinnula *et al*., 1991). Moreover, recent studies have shown that levels of hydrogen peroxide produced at the airway surface from normal airway epithelial cells are enough to support production of bacteriocidal hypothiocyanate,

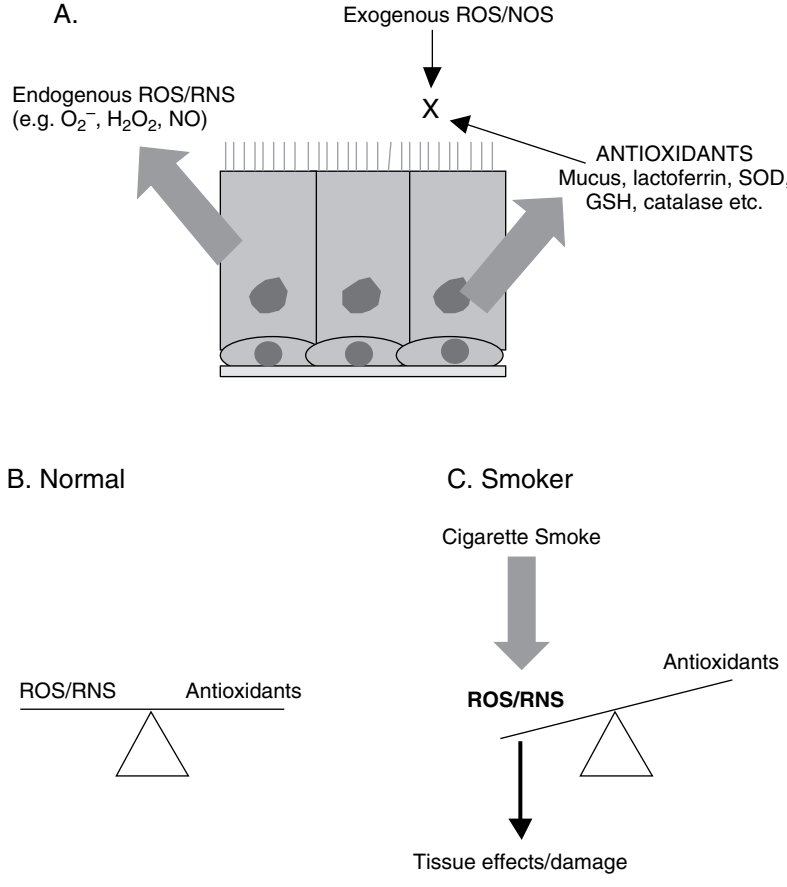


Figure 16.3 Production of reactive oxygen and nitrogen species, as well as antioxidants, by the epithelium. (A) Epithelial cells endogenously produce reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), such as nitric oxide (NO). In addition, they release a range of antioxidants that can help regulate the activity of both endogenous and exogenous ROS/RNS. These include mucins, lactoferrins, superoxide dismutases (SOD), glutathione (GSH) and catalase. (B) In the normal airway, generation of antioxidants is sufficient to prevent excessive effects of ROS/RNS and normal airway function is maintained. (C) In smokers, the huge additional burden of ROS/RNS derived from cigarette smoke can overwhelm the actions of antioxidants leading to chronic effects of ROS/NOS on cellular functions and tissue damage

while production was markedly impaired using epithelial cells derived from patients with cystic fibrosis (Moskwa *et al*., 2007). This generation of hydrogen peroxide appeared to be mediated mainly by Duox enzymatic activity (Moskwa *et al*., 2007). It should be noted that epithelial production of hydrogen peroxide can be increased by a variety of pro-inflammatory stimuli, such as PAF and TNF-α (Rochelle *et al*., 1998). Reactive oxygen species have marked effects on the functions of airway cells, including epithelial cells. Oxidant stress is known to play a role, for example, in epithelial production of several cytokines, chemokines, and adhesion molecules (Martin *et al*., 1998; Nakanaga *et al*., 2007), most likely via the ability of ROS to alter the expression and activation of transcription factors, such as NF-κB, and AP-1, that are known to play a role in induction of these molecules.

Among the reactive nitrogen species produced by epithelial cells, nitric oxide (NO) is the most studied (Figure 16.3(A)). NO is a highly reactive free radical with wide-ranging biological effects on multiple target cells. In general, it exerts its effects by three main categories of molecular actions: (1) via donation of electrons to transition metals forming metal-nitrosyl complexes that can activate or inhibit the function of proteins. A well-characterized example of this is the activation of soluble guanylyl cyclase, which contains a heme group. NO disrupts the bond between the ferrous iron of this heme group and histidine 105 of the enzyme leading to increased cGMP synthesis; (2) via S-nitrosylation of thiol groups in amino acid constituents of proteins or peptides; and (3) via reactions with other radicals, such as superoxide, leading to the formation of peroxynitrite, which can oxidize thiol residues in amino acids, leading to altered biological functions.

NO is produced from L-arginine by any of three isoforms of the enzyme, nitric oxide synthase (NOS). Although airway epithelial cells have been reported to express mRNA for all three isoforms of NOS, the Type II, or inducible form, of the enzyme (iNOS) is the major form detected at the protein level (Guo *et al*., 1995). It has been suggested that epithelial iNOS activity is the major determinant of levels of NO in exhaled breath (Lane *et al*., 2004), and epithelial expression of iNOS, and production of NO, in vitro is increased upon exposure to a variety of pro-inflammatory stimuli, including cytokines, and infection with any of a number of respiratory viruses (Proud, 2005). Levels of exhaled NO are altered in several inflammatory airway diseases. Increased levels of exhaled NO are seen in asthma, chronic obstructive pulmonary disease, and respiratory viral infections (Kharitonov *et al*., 1994, 1995; Maziak *et al*., 1998), leading to the suggestion that NO may contribute to disease pathogenesis and be a marker of inflammation. By contrast, however, levels of exhaled NO are decreased in other inflammatory disorders, such as chronic rhinosinusitis (Lindberg *et al*., 1997), and epithelial iNOS expression and NO production also are decreased in patients with cystic fibrosis. Thus, caution is needed in associating the presence of increased NO with a causative role in pathogenesis.

The initial focus on NO in the airway was as a pro-inflammatory and deleterious mediator, based on its actions as a vasodilator that can enhance vascular permeability under some conditions, and its ability to induce mucus secretion, modulation of ion transport, enhancement of COX-2 activity and prostaglandin production, and to cause tissue damage via formation of peroxynitrite (Bove and van der Vliet, 2006). By contrast, NO can also exert several positive effects. It is a bronchodilator, positively regulates ciliary beat frequency, inhibits selective adhesion processes necessary for inflammatory cell recruitment, and also inhibits the increased vascular permeability seen at inflammatory sites (Bove and van der Vliet, 2006; Granger and Kubes, 1996). NO also plays an important role in host defence. It displays direct antiviral effects against several important respiratory viruses, including

rhinoviruses, respiratory syncytial virus and influenza (Proud, 2005). It can also suppress viral induction of a range of cytokines and chemokines (Sanders *et al*., 1998). This is, perhaps, not surprising, as NO is known to modulate several key signalling pathways that have been implicated in cytokine induction. These include members of the mitogen-activated protein kinase family that have been implicated in both transcriptional and posttranscriptional regulation of virally-induced cytokines, as well as members of the Janus family of kinases (Schindler and Bogdan, 2001). NO also can nitrosylate a variety of transcription factors, such as SP1 and EGR-1, that contain Cys_2His_2 zinc finger type DNA-binding motifs, as well as transcription factors such as NF-κB and AP-1, that contain cysteine residues close to their DNA binding regions (Bove and van der Vliet, 2006). Interestingly, in light of these observations, a recent study of in vivo experimental rhinovirus infections found not only that levels of epithelial iNOS expression correlated with levels of exhaled NO, but that subjects with the highest levels of exhaled NO had lower symptom scores and cleared virus more rapidly (Sanders *et al*., 2004). It also has been suggested that defective generation of NO in subjects with cystic fibrosis contributes to the increased susceptibility of such patients to repeated airway infections (Zheng *et al*., 2003). Thus, the overall role of NO in airway diseases is complex and incompletely understood.

16.6 Epithelial production of proteases

Proteases in the airway can have profound effects on epithelial function and on other airway structural cells to modulate airway inflammation. They can also affect airway structural/matrix proteins which appear to be critical to the pathogenesis of airway remodelling. Although proteases can be derived from inhaled pathogens and from inflammatory cells, it is now recognized that the epithelial cell is a significant source of proteases that can modulate the environment in the airway. Although the epithelium is the source of a variety of proteases, such as cathepsin B and members of the a disintegrin and metalloproteinase (ADAM) family of enzymes, particular interest has focused on members of the family of zinc-dependent matrix metalloproteinases (MMPs) which collectively can degrade all components of the extracellular matrix, and are thought to be central to the pathogenesis of COPD and airway remodelling in asthma. Airway epithelial cells produce MMP-2 (collagenase A), MMP-7 (matrilysin), MMP-9 (gelatinase B) and MMP-12 (metalloelastase). Levels of MMP2, MMP-9 and MMP-12 are all increased in asthmatic subjects, but MMP-9 appears to be the major MMP in the airway of asthmatics (Kelly and Jarjour, 2003). MMP-9 is strongly expressed in repairing epithelial cells and can be induced by pro-inflammatory cytokines and by activation of epithelial proteinase-activated receptor (PAR)-2. Levels of MMP-9 are increased in blood, sputum, BAL and airway biopsies of asthmatic subjects and in BAL after allergen challenge. The ratio of MMP-9 to its primary inhibitor, tissue inhibitor of metalloproteinases (TIMP)-1, is selectively increased by some pro-inflammatory stimuli and is also increased in the airway during acute asthma exacerbations (Cohn *et al*., 2004). This increased activity of MMP-9 to TIMP-1 enhances effects on matrix proteins and favors airway remodelling. MMP-9 can also activate TGFβ1, which is a key regulator of fibrotic pathways. Other MMPs also have the capacity to modulate inflammation and remodelling. MMP-12 regulates subepithelial fibrosis and eosinophilia in mouse models, while MMP-2 appears to regulate inflammatory cell migration within the lung (Cohn *et al*., 2004). Although epithelial cells produce MMPs that can degrade matrix proteins, it should be noted that they

can also contribute to matrix protein deposition in the airway. They produce fibronectin and tenascin, and production of these proteins is markedly upregulated at sites of epithelial injury and repair.

16.7 The role of epithelial cells in the recruitment of inflammatory cells

The mechanisms by which leukocytes interact with, and migrate through the epithelium remain relatively poorly understood. It is obvious, however, that recruitment of inflammatory cells into the airway is dependent on the presence of various chemoattractants, including arachidonic acid metabolites, cytokines, chemokines, and peptides. Production of these chemoattractants by airway epithelial cells is considered to play a critical function in the recruitment of inflammatory cells into the airway. In addition to chemoattractant stimuli, however, migration of inflammatory cells to the airway epithelium and the airway lumen requires specific interactions of leukocyte counterligands with adhesion molecules on the epithelium. Epithelial cells do not express E-selectin, and it is controversial as to whether vascular cell adhesion molecule-1 (VCAM-1) is expressed. The role of epithelial cell adhesion molecule (Ep-CAM), if any, in leukocyte–epithelial interactions is unknown. However, airway epithelial cells do express intercellular adhesion molecule-1 (ICAM-1/CD54), and expression is enhanced following exposure to pro-inflammatory cytokines, as well as in response to respiratory viral infections. Epithelial cells of asthmatics show increased expression of ICAM-1 compared with normal subjects (Vignola *et al*., 1993). ICAM-1 is a counterligand for leukocyte β_2 integrins (CD11/CD18), which play a central role in leukocyte-epithelial adhesion, as preincubation of leukocytes with monoclonal antibodies to CD18 abrogates this adhesion. Interestingly, blockage of epithelial ICAM-1 is much less effective in reducing leukocyte adhesion, implying that additional CD18-dependent, ICAM-1-independent, adhesion mechanisms must also exist (Tosi *et al*., 1994). Further support for this concept comes from studies in mice, showing that neutrophil emigration into the alveolar spaces during acute *Streptococcus pneumoniae* infection remained normal in animals in which the genes for both P-selectin and ICAM-1 were mutated (Bullard *et al*., 1995). Recent studies have also examined transepithelial migration of leukocytes in the physiologically relevant basolateral to luminal direction. Again, interactions between epithelial ICAM-1 and β_2 integrins contribute to this process but blockade of either ICAM-1 or of β_2 integrins does not lead to complete inhibition (Kidney and Proud, 2000).

In terms of alternative epithelial adhesion molecules, there is evidence that β_2 integrins bind to oligosaccharide determinants on epithelial cells. For example, heparin and heparin sulfate proteoglycans can bind CD11/CD18, although whether this is a major event on epithelial cells is unclear. Fucosylated proteoglycans also bind to β_2 integrins and play a role in neutrophil adhesion to intestinal epithelial cells, but these data have not yet been extended to airway epithelial cells (Zen and Parkos, 2003). The junctional adhesion molecule, JAM-C, is a component of epithelial desmosomes which binds specifically to CD11b/CD18 on neutrophils. JAM-C gene deficient mice show altered airway responsiveness and increased numbers of circulating granulocytes (Imhof *et al*., 2007). In addition, antibodies to JAM-C inhibit neutrophil transmigration through intestinal epithelial cells, but such studies have not yet been extended to airway epithelium (Zen *et al*., 2004).

16.8 Anti-inflammatory actions of epithelial cells

The capacity of the epithelium to produce the products described above indicate that epithelial cells can be a major contributor to airway inflammation and structural remodelling. However, in general, the function of the epithelium is to contribute to maintaining normal airway function. In this context, the epithelial cell plays a role in limiting excessive airway inflammation by contributing to the catabolism of some proinflammatory mediators and by inhibiting the actions of others. Two major classes of epithelial products that are important in this regard are inhibitors of proteases and peptides, and antioxidants.

16.8.1 Production of protease inhibitors and peptidases

As noted above, the airway epithelium is, itself, a source of several proteases that can exert functional effects in the airway. In addition, however, proteases derived from other host cells, such as neutrophils, or from inhaled pathogens, including a wide array of allergens, such as house dust mite, cockroach, fungi, and some pollens, can exert a variety of actions on target tissues within the airway, including the epithelium itself. For example, proteases derived from several allergens, as well as neutrophil elastase, induce production of cytokines and chemokines from epithelial cells, while neutrophil elastase is also a stimulus for epithelial mucin production (Shao and Nadel, 2005). Moreover, elastase, cathepsin G and several allergen-derived proteases can compromise epithelial barrier function via disruption of cell–cell contacts, thereby enhancing access of allergens to underlying antigen-presenting cells (see Chapter 15). Indeed, studies in mice have revealed that proteolytic activity is an important factor in host sensitization toward allergens (Kheradmand *et al*., 2002; Fattouh *et al*., 2005).

It is clear, therefore, that while recruitment of neutrophils and other inflammatory cells is an important component of the host response to inhaled pathogens, excessive exposure of the airway to proteases derived from pathogens or inflammatory cells can have deleterious effects on airway function. To help protect the airway from the negative effects of proteases, epithelial cells produce a range of protease inhibitors. Although epithelial cells themselves produce MMP-2 and MMP-9 that can metabolize matrix proteins (see above), the epithelium is also a source of the major inhibitor of these enzymes, TIMP-1. Under normal circumstances, the ratio of MMP to TIMP-1 is approximately 1:1 but several pro-inflammatory stimuli can differentially regulate this ratio to promote or reduce matrix degradation (Yao *et al*., 1997). Epithelial cells also produce a range of inhibitors to reduce the activity of serine and cysteine proteinases. These include secretory leukocyte protease inhibitor (SLPI) and elafin that are able to regulate the effects of neutrophil elastase on cytokine production, glycoconjugate production and downstream tissue damage. Administration of SLPI, the major elastase inhibitor in the large airway, to patients with cystic fibrosis (CF) reduces both elastase activity and IL-8 levels in airway secretions (McElvaney *et al*., 1992). SLPI is a broad-ranging inhibitor that can limit the actions of multiple proteases, including cathepsin G, mast cell chymase, and enzymes with tryptic and chymotryptic specificity. By contrast, Elafin, also called elastase-specific inhibitor (ESI), selectively inhibits both elastase and proteinase 3. The broad-ranging inhibitory function of epithelial cells is further enhanced by production of cystatin C, α_1-antiprotease inhibitor, and α_1-antichymotrypsin. Expression of several of these inhibitors increases in response to pro-inflammatory cytokines (Sallenave *et al*., 1994).

Epithelial cells also degrade and regulate the actions of several biologically active peptides produced as part of the airway inflammatory response because they express cell-surface peptidases, such as neutral endopeptidase and aminopeptidase M (Proud *et al*., 1994), that can degrade bradykinin, enkephalins and several other peptides.

16.8.2 Antioxidant production

As noted above, reactive oxygen species (ROS) can exert profound effects in the airway. In the normal lung, the airway have an effective protection capacity against ROS, with the epithelial cell being a significant contributor to these protective mechanisms via production of a range of antioxidants (Figure 16.3 (A) and (B)). Studies on animal models have shown that mucins provide significant protection against oxidants, and that epithelial production of these glycoproteins is increased upon oxidant exposure (Cross *et al*., 1984; Adler and Li, 2001). In addition, the epithelium produces molecules, such as lactoferrin, that can bind free iron and so could be protective, as metal ions including iron are important in the generation of oxidant radicals via the Fenton reaction. The primary protection against the activity of superoxide radicals are members of the superoxide dismutase (SOD) enzyme family, including manganese SOD, copper/zinc SOD and extracellular SOD, all of which are widely expressed in the lung (Kinnula and Crapo, 2003). Epithelial cells produce both manganese SOD and copper/zinc SOD, which can be induced by several cytokines and are generally thought to act as important bulk scavengers of superoxide. Epithelial lining fluid also contains a high content of the sulphydryl-containing antioxidant glutathione (GSH), a molecule that also has been considered as one of the primary antioxidants in the human lung (Cantin *et al*., 1987). Other antioxidants produced by airway epithelial cells include catalase and enzymes associated with GSH metabolism. The latter group of enzymes includes glutathione peroxidases (GPXs), glutathione reductase, and glutathione synthase. Catalase and the enzymes associated with GSH metabolism are involved in hydrogen peroxide metabolism, and due to the high levels of enzymes associated with GSH metabolism in the epithelial lining fluid, these enzymes are thought to have central roles in lung protection.

Although the airway contains sufficient antioxidant protection under normal circumstances, cigarette smoke contains an enormous burden of oxygen free radicals and high concentrations of NO, both of which can overwhelm normal defence mechanisms to cause significant oxidant stress within the airway (Figure 16.3 (B) and (C)). Some antioxidant enzymes are induced, but the extent of induction is insufficient to protect the airway epithelium and other lung tissues against the deleterious effects of cigarette smoke. Impaired oxidant–antioxidant balance is thought to be a major factor in the pathogenesis of smoking-related COPD and functional polymorphisms in enzymes such as SOD may be a contributing factor to the individual susceptibility to smoking-related lung disease (Kinnula, 2005). Consequently, pharmacological approaches to enhance antioxidant protection have been considered of potential therapeutic benefit. Variable results have been obtained administering vitamins with antioxidant functions to patients with COPD. N-acetylcysteine (NAC), which may enhance glutathione activity, has shown positive effects in animal models and has been tested in humans with some benefits, but NAC can also have pro-oxidant side effects. Similarly, synthetic compounds with superoxide dismutase and catalase activities have shown promising results in animal models against a variety of oxidant exposures including cigarette smoke in the lung but additional studies are needed to assess effectiveness in humans (Kinnula, 2005).

16.9 Summary

The epithelial cell can no longer be considered primarily in terms of its function as a barrier. Rather, it generates a wide variety of products that can modulate numerous aspects of airway function. It can regulate the content of airway secretions, control inflammatory cell recruitment to the airway, and modulate airway tone and vascular permeability. Importantly, it is an important contributor to host immune responses, and a regulator of airway remodelling. As such, the airway epithelium almost certainly contributes to the pathogenesis of the allergic inflammatory response of asthma, as well as the inflammatory responses seen in COPD, infective bronchitis and bronchiectasis. Given that over-exuberant inflammatory responses of the epithelial cell can have profound effects on airway function, it is clearly a primary target for novel anti-inflammatory interventions in airway disorders, particularly those drugs administered by inhalation. Thus, a better understanding of the dynamic properties of the airway epithelium during acute inflammatory events may provide additional insights into appropriate targets for therapeutic intervention.

Acknowledgements

The authors acknowledge support from the Canadian Institutes of Health Research and the Alberta Heritage Foundation for Medical Research. Dr Leigh holds the GlaxoSmithKline/CIHR Professorship in Inflammatory Lung Diseases. Dr Proud is the recipient of a Canada Research Chair in Inflammatory Airway Diseases.

References

Ackerman, V., Carpi, S., Bellini, A., Vassalli, G., Marini, M. & Mattoli, S. (1995) Constitutive expression of endothelin in bronchial epithelial cells of patients with symptomatic and asymptomatic asthma and modulation by histamine and interleukin-1. *J. Allergy Clin. Immunol*., **91**, 618–627.

Adler, K. B. & Li, Y. (2001) Airway epithelium and mucus: intracellular signaling pathways for gene expression and secretion. *Am. J. Respir. Cell Mol. Biol*., **25**, 397–400.

Allen, S. J., Crown, S. E. & Handel, T. M. (2007) Chemokine:receptor structure, interactions and antagonism. *Annu. Rev. Immunol*., **25**, 787–820.

Beck, L. A., Tancowny, B., Brummet, M. E., Asaki, S. Y., Curry, S. L., Penno, M. B., Foster, M., Bahl, A. & Stellato, C. (2006) Functional analysis of the chemokine receptor CCR3 on airway epithelial cells. *J. Immunol*., **177**, 3344–3354.

Behera, A. K., Kumar, M., Matsuse, H., Lockey, R. F. & Mohapatra, S. S. (1998) Respiratory syncytial virus induces the expression of 5-lipoxygenase and endothelin-1 in bronchial epithelial cells. *Biochem. Biophys. Res. Commun*., **251**, 704–709.

Bochner, B. S., Hudson, S. A., Xiao, H. Q. & Liu, M. C. (2003) Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions. *J. Allergy Clin. Immunol*., **112**, 930–934.

Bonfield, T. L., Konstan, M. W., Burfeind, P., Panuska, J. R., Hilliard, J. B. & Berger, M. (1995) Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol*., **13**, 257–261.

Bove, P. F. & van der Vliet, A. (2006) Nitric oxide and reactive nitrogen species in airway epithelial cell signaling and inflammation. *Free Radic. Biol. Med*., **41**, 515–527.

Bullard, D. C., Qin, L., Lorenzo, I., Quinlin, W. M., Doyle, N. A., Bosse, R., Vestweber, D., Doerschuk, C. M. & Beaudet, A. L. (1995) P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. *J. Clin. Invest*., **95**, 1782–1788.

Cantin, A. M., North, S. L., Hubbard, R. C. & Crystal, R. G. (1987) Normal alveolar epithelial lining fluid contains high levels of glutathione. *J. Appl. Physiol*., **63**, 152–157.

Chung, K. F. & Barnes, P. J. (1999) Cytokines in asthma. *Thorax*, **54**, 825–857.

Churchill, L., Chilton, F. H., Resau, J. H., Bascom, R., Hubbard, W. C. & Proud, D. (1989) Cyclooxygenase metabolism of endogenous arachidonic acid by cultured human tracheal epithelial cells. *Am. Rev. Respir. Dis*., **140**, 449–459.

Cohn, L., Elias, J. A. & Chupp, G. L. (2004) Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol*., **22**, 789–815.

Colobran, R., Pujol-Borrell, R., Armengol, M. P. & Juan, M. (2007) The chemokine network. I. How the genomic organization of chemokines contains clues for deciphering their functional complexity. *Clin. Exp. Immunol*., **148**, 208–217.

Contoli, M., Message, S. D., Laza-Stanca, V., Edwards, M. R., Wark, P. A. B., Bartlett, N. W., Kebadze, T., Mallia, P., Stanciu, L. A., Parker, H. L., Slater, L., Lewis-Antes, A., Kon, O. M., Holgate, S. T., Davies, D. E., Kotenko, S. V., Papi, A. & Johnston, S. L. (2006) Role of deficient type-III interferon-l production in asthma exacerbations. *Nature Medicine*, **12**, 1023–1026.

Cromwell, O., Hamid, Q., Corrigan, C. J., Barkans, J., Meng, Q., Collins, P. D. & Kay, A. B. (1992) Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1β and tumour necrosis factor-α. *Immunology*, **77**, 330–337.

Cross, C. E., Halliwell, B. & Allen, A. (1984) Antioxidant protection: a function of tracheobronchial and gastrointestinal mucus. *Lancet*, **1**, 1328–1330.

DiCosmo, B. F., Geba, G. P., Picarella, D., Elias, J. A., Rankin, J. A., Stripp, B. R., Whitsett, J. A. & Flavell, R. A. (1994) Airway epithelial cell expression of interleukin-6 in transgenic mice: uncoupling of airway inflammation and bronchial hyperreactivity. *J. Clin. Invest*., **94**, 2028–2035.

Eddleston, J., Christiansen, S. C. & Zuraw, B. L. (2002) Functional expression of the C-X-C chemokine receptor CXCR4 by human bronchial epithelial cells: regulation by proinflammatory mediators. *J. Immunol*., **169**, 6445–6451.

Einarsson, O., Geba, G. P., Zhu, Z., Landry, M. & Elias, J. A. (1996) Interleukin-11: stimulation in vivo and in vitro by respiratory viruses and induction of airways hyperresponsiveness. *J. Clin. Invest*., **97**, 915–924.

Fattouh, R., Pouladi, M. A., Alvarez, D., Johnson, J. R., Walker, T. D., Goncharova, S., Inman, M. D. & Jordana, M. (2005) House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am. J. Respir. Crit. Care Med*., **172**, 314–321.

Fernandes, L. B., Henry, P. J., Rigby, P. J. & Goldie, R. G. (1996) Endothelin B (ETB) receptor-activated potentiation of cholinergic nerve-mediated contraction in human bronchus. *Br. J. Pharmacol*., **118**, 1873–1874.

Fujimoto, K., Imaizumi, T., Yoshida, H., Takanashi, S., Okumura, K. & Satoh, K. (2001) Interferon-γ stimulates fractalkine expression in human bronchial epithelial cells and regulates mononuclear cell adherence. *Am. J. Respir. Cell Mol. Biol*., **25**, 233–238.

Geiszt, M., Witta, J., Baffi, J., Lekstrom, K. & Leto, T. L. (2003) Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J*., **17**, 1502–1504.

Goldie, R. G. & Henry, P. J. (1999) Endothelins and asthma. *Life Sci*., **65**, 1–15.

Granger, D. N. & Kubes, P. (1996) Nitric oxide as an antiinflammatory agent. *Methods Enzymol*., **269**, 434–442.

Guo, F. H., De Raeve, H. R., Rice, T. W., Stuehr, D. J., Thunnissen, F. B. J. M. & Erzurum, S. C. (1995) Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl Acad. Sci. USA*, **92**, 7809–7813.

Heijink, I. H., Kies, M., Van Oosterhout, A. J. M., Postma, D. S., Kauffman, H. F. & Vellebga, E. (2007) Der p, IL-4, and TGF-β cooperatively induce EGFR-dependent TARC expression in airway epithelium. *Am. J. Respir. Cell Mol. Biol*., **36**, 351–359.

Holtzman, M. J. (1992) Arachidonic acid metabolism in airway epithelial cells. *Annu. Rev. Physiol*., **54**, 303–329.

Holtzman, M. J., Ferdman, B., Bohrer, A. & Turk, J. (1991) Synthesis of the 1-O-hexadecyl molecular species of platelet-activating factor by airway epithelial and vascular endothelial cells. *Biochem. Biophys. Res. Commun*., **177**, 357–364.

Huston, D. P. & Liu, Y. J. (2006) Thymic stromal lymphopoietin: a potential therapeutic target for allergy and asthma. *Curr. Allergy Asthma Rep*., **6**, 372–376.

Imhof, B. A., Zimmerli, C., Gliki, G., Ducrest-Gay, D., Juillard, P., Hammel, P., Adams, R. & Aurrand-Lions, M. (2007) Pulmonary dysfunction and impaired granulocyte homeostasis result in poor survival of Jam-C-deficient mice. *J. Pathol*., **212**, 198–202.

Jacobs, E. R. & Zeldin, D. C. (2001) The lung HETEs (and EETs) up. *Am. J. Physiol*., **280**, H1–H10.

Kang, J. X., Man, S. F., Hirsch, A. J. & Clandinin, M. T. (1994) Characterization of platelet-activating factor binding to human airway epithelial cells: modulation by fatty acids and ion-channel blockers. *Biochem. J*., **303**, 795–802.

Kelly, E. A. & Jarjour, N. N. (2003) Role of matrix metalloproteinases in asthma. *Curr. Opin. Pulm. Med*., **9**, 28–33.

Kelly, M. M., Leigh, R., Bonniaud, P., Ellis, R., Wattie, J., Smith, M. J., Martin, G., Panju, M., Inman, M. D. & Gauldie, J. (2005) Epithelial expression of profibrotic mediators in a model of allergen-induced airway remodeling. *Am. J. Respir. Cell Mol. Biol*., **32**, 99–107.

Kelsen, S. G., Aksoy, M. O., Yang, Y., Shahabuddin, S., Litvin, J., Safadi, F. & Rogers, T. J. (2004) The chemokine receptor CXCR3 and its splice variant are expressed in human airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **287**, L584–L591.

Kenney, J. S., Baker, C., Welch, M. R. & Altman, L. C. (1994) Synthesis of interleukin-1α, interleukin-6, and interleukin-8 by cultured human nasal epithelial cells. *J. Allergy Clin. Immunol*., **93**, 1060–1067.

Kharitonov, S. A., Yates, D., Robbins, R. A., Logan-Sinclair, R., Shinebourne, E. & Barnes, P. J. (1994) Increased nitric oxide in exhaled air of asthmatic patients. *Lancet*, **343**, 133–135.

Kharitonov, S. A., Yates, D. & Barnes, P. J. (1995) Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. *Eur. Respir. J*., **8**, 295–297.

Kheradmand, F., Kiss, A., Xu, J., Lee, S. H., Kolattukudy, P. E. & Corry, D. B. (2002) A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J. Immunol*., **169**, 5904–5911.

Kidney, J. C. & Proud, D. (2000) Neutrophil transmigration across human airway epithelial monolayers; mechanisms and dependence on electrical resistance. *Am. J. Respir. Cell Mol. Biol*., **23**, 389–395.

Kim, C. H., Rott, L., Kunkel, E. J., Genovese, M. C., Andrew, D. P., Wu, L. & Butcher, E. C. (2001) Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest*., **108**, 1331–1339.

King, C., Brennan, S., Thompson, P. J. & Stewart, G. A. (1998) Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J. Immunol*., **161**, 3645–3651.

Kinnula, V. L. (2005) Focus on antioxidant enzymes and antioxidant strategies in smoking related airway diseases. *Thorax*, **60**, 693–700.

Kinnula, V. L. & Crapo, J. D. (2003) Superoxide dismutases in the lung and human lung diseases. *Am. J. Respir. Crit. Care Med*., **167**, 1600–1619.

Kinnula, V. L., Everitt, J. I., Whorton, A. R. & Crapo, J. D. (1991) Hydrogen peroxide production by alveolar type II cells, alveolar macrophages, and endothelial cells. *Am. J. Physiol*., **261**, L84–L91.

Kishimoto, T., Akira, S. & Taga, T. (1992) Interleukin-6 and its receptor: a paradigm for cytokines. *Science*, **258**, 593–597.

Knight, D. A. & Holgate, S. T. (2003) The airway epithelium: structural and functional properties in health and disease. *Respirology*, **8**, 432–446.

Kumlin, M., Hamberg, M., Granström, E., Björk, T., Dahlén, B., Matsuda, H., Zetterström, O. & Dahlén, S.-E. (1990) 15(S)-hydroxyeicosatetraenoic acid is the major arachidonic acid metabolite in human bronchi: association with airway epithelium. *Arch. Biochem. Biophys*., **282**, 254–262.

Lai, C. K. W., Polosa, R. & Holgate, S. T. (1990) Effect of 15-(s)-hydroxyeicosatetraenoic acid on allergen-induced asthmatic responses. *Am. Rev. Respir. Dis*., **141**, 1423–1427.

Lane, C., Knight, D., Burgess, S., Franklin, P., Horak, F., Legg, J., Moeller, A. & Stick, S. (2004) Epithelial inducible nitric oxide synthase activity is the major determinant of nitric oxide concentration in exhaled breath. *Thorax*, **59**, 757–760.

Lindberg, S., Cervin, A. & Runer, T. (1997) Nitric oxide (NO) production in the upper airways is decreased in chronic sinusitis. *Acta Otolaryngol. (Stockh.)*, **117**, 113–117.

Liu, M. C., Hubbard, W. C., Proud, D., Stealey, B. A., Galli, S. J., Kagey-Sobotka, A., Bleecker, E. R. & Lichtenstein, L. M. (1991) Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics: cellular, mediator, and permeability changes. *Am. Rev. Respir. Dis*., **144**, 51–58.

Marini, M., Vittori, E., Hollemborg, J. & Mattoli, S. (1992) Expression of the potent inflammatory cytokines, granulocyte-macrophage colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J. Allergy Clin. Immunol*., **89**, 1001–1009.

Markewitz, B. A., Kohan, D. E. & Michael, J. R. (1995) Endothelin-1 synthesis, receptors, and signal transduction in alveolar epithelium: evidence for an autocrine role. *Am. J. Physiol*., **268**, L192–L200.

Martin, L. D., Krunkosky, T. M., Voynow, J. A. & Adler, K. B. (1998) The role of reactive oxygen and nitrogen expression in airway epithelial gene expression. *Environ. Health Perspect*., **106** (Suppl 5), 1197–1203.

Mattoli, S., Mattoso, V. L., Soloperto, M., Allegra, L. & Fasoli, A. (1991) Cellular and biochemical characteristics of bronchoalveolar lavage fluid in symptomatic nonallergic asthma. *J. Allergy Clin. Immunol*., **87**, 794–802.

Maziak, W., Loukides, S., Culpit, S., Sullivan, P., Kharitonov, S. A. & Barnes, P. J. (1998) Exhaled nitric oxide in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med*., **157**, 998–1002.

McElvaney, N. G., Nakamura, H., Birrer, P., Hébert, C. A., Wong, W. L., Alphonso, M., Baker, J. B., Catalano, M. A. & Crystal, R. G. (1992) Modulation of airway inflammation in cystic fibrosis. In vivo suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor. *J. Clin. Invest*., **90**, 1296–1301.

Melillo, E., Wooley, K. L., Manning, P. J., Watson, R. M. & O'Byrne, P. M. (1994) Effect of inhaled PGE_2 on exercise-induced bronchoconstriction in asthmatic subjects. *Am. J. Respir. Crit. Care Med*., **149**, 1138–1141.

Moskwa, P., Lorentzen, D., Excoffon, K. J. D. A., Zabner, J., McCray, P. B., Nauseef, W. M., C., D. & Bánfi, B. (2007) A novel host defense system of airways is defective in cystic fibrosis. *Am. J. Respir. Crit. Care Med*., **175**, 174–183.

Nakanaga, T., Nadel, J. A., Ueki, I. F., Koff, J. L. & Shao, M. X. G. (2007) Regulation of interleukin-8 via an airway epithelial signaling cascade. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **292**, L1289–L1296.

Nomura, A., Uchida, Y., Kameyama, M., Saotome, M., Oki, K. & Hasegawa, S. (1989) Endothelin and bronchial asthma. *Lancet*, **1**, 747–748.

Nonaka, M., Nonake, R., Jordana, M. & Dolovich, J. (1996) GM-CSF, IL-8, IL-1R, TNF-αR, and HLA-DR in nasal epithelial cells in allergic rhinitis. *Am. J. Respir. Crit. Care Med*., **153**, 1675–1681.

Otsuka, H., Kusumi, T., Kanai, S., Koyama, M., Kuno, Y. & Takizawa, R. (1998) Stem cell factor mRNA expression and production in human nasal epithelial cells: contribution to the accumulation of mast cells in the nasal epithelium of allergy. *J. Allergy Clin. Immunol*., **102**, 757–764.

Panina-Bordignon, P., Papi, A., Mariani, M., Di Lucia, P., Casoni, G., Bellettato, C., Buonsanti, C., Miotto, D., Mapp, C., Villa, A., G., A., Fabbri, L. M. & Sinigaglia, F. (2001) The C-C chemokine

receptors CCR4 and CCR8 identify airway T cells of allergen challenged atopic asthmatics. *J. Clin. Invest*., **107**, 1357–1364.

Pavord, I. D., Wong, C. S., Williams, J. & Tattersfield, A. E. (1993) Effect of inhaled prostaglandin E_2 on allergen-induced asthma. *Am. Rev. Respir. Dis*., **148**, 87–90.

Petkova, D. K., Pang, L., Range, S. P., Holland, E. & Knox, A. J. (1999) Immunocytochemical localization of cyclo-oxygenase isoforms in cultured human airway structural cells. *Clin. Exp. Allergy*, **29**, 965–972.

Polosa, R., Puddicombe, S. M., Krishna, M. T., Tuck, A. B., Howarth, P. H., Holgate, S. T. & Davies, D. E. (2002) Expression of c-erbB receptors and ligands in the bronchial epithelium of asthmatic subjects. *J. Allergy Clin. Immunol*., **109**, 75–81.

Proud, D. (2005) Nitric oxide and the common cold. *Curr. Opin. Allergy Clin. Immunol*., **5**, 37–42.

Proud, D., Subauste, M. C. & Ward, P. E. (1994) Glucocorticoids do not alter peptidase expression on a human bronchial epithelial cell line. *Am. J. Respir. Cell Mol. Biol*., **11**, 57–65.

Psarras, S., Volonaki, E., Skevaki, C. L., Xatzipsalti, M., Bossios, A., Pratsinis, H., Tsigkos, S., Gourgiotis, D., Constantopoulos, A. G., Papapetropoulos, A., Saxoni-Papageorgiou, P. & Papadopoulos, N. G. (2006) Vascular endothelial growth factor-mediated induction of angiogenesis by human rhinovirus. *J. Allergy Clin. Immunol*., **117**, 291–297.

Ray, P., Tang, W., Wang, P., Homer, R., Kuhn, C. III, Flavell, R. A. & Elias, J. A. (1997) Regulated overexpression of interleukin-11 in the lung. Use to dissociate development-dependent and -independent phenotypes. *J. Clin. Invest*., **100**, 2501–2511.

Redington, A. E., Meng, Q.-H., Springall, D. R., Evans, T. J., Créminon, C., Maclouf, J., Holgate, S. T., Howarth, P. H. & Polak, J. M. (2001) Increased expression of inducible nitric oxide synthase and cyclo-oxygenase-2 in the airway epithelium of asthmatic subjects and regulation by corticosteroid treatment. *Thorax*, **56**, 351–357.

Reibman, J., Hsu, Y., Chen, L. C., Bleck, B. & Gordon, T. (2003) Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am. J. Respir. Cell Mol. Biol*., **28**, 648–654.

Riccio, M. M., Reynolds, C. J., Hay, D. W. & Proud, D. (1995) Effects of intranasal administration of endothelin-1 to allergic and nonallergic individuals. *Am. J. Respir. Crit. Care Med*., **152**, 1757–1764.

Rimaniol, A. C., Till, S. J., Garcia, G., Capel, F., Godot, V., Balabanian, K., Durand-Gasselin, I., Varga, E. M., Simonneau, G., Emilie, D., Durham, S. R. & Humbert, M. (2003) The CX3C chemokine fractalkine in allergic asthma and rhinitis. *J. Allergy Clin. Immunol*., **112**, 1139–1146.

Rochelle, L. G., Fischer, B. M. & Adler, K. B. (1998) Concurrent production of reactive oxygen and nitrogen species by airway epithelial cells in vitro. *Free Radic. Biol. Med*., **24**, 863–868.

Salari, H. & Schellenberg, R. R. (1991) Stimulation of human airway epithelial cells by platelet activating factor (PAF) and arachidonic acid produces 15-hydroxyleicosatetraenoic acid (15-HETE) capable of contracting bronchial smooth muscle. *Pulm. Pharmacol*., **4**, 1–7.

Sallenave, J.-M., Shulmann, J., Crossley, J., Jordana, M. & Gauldie, J. (1994) Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am. J. Respir. Cell Mol. Biol*., **11**, 733–741.

Sanders, S. P., Siekierski, E. S., Porter, J. D., Richards, S. M. & Proud, D. (1998) Nitric oxide inhibits rhinovirus-induced cytokine production and viral replication in a human respiratory epithelial cell line. *J. Virol*., **72**, 934–942.

Sanders, S. P., Proud, D., Siekierski, E. S., Yachechko, R. & Liu, M. C. (2004) Role of nasal nitric oxide in the resolution of experimental rhinovirus infection. *J. Allergy Clin. Immunol*., **113**, 697–702.

Schindler, H. & Bogdan, C. (2001) NO and a signalling molecule: effects on kinases. *Int. Immunopharmacol*., **1**, 1443–1455.

Shao, M. X. & Nadel, J. A. (2005) Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species and TNF-α-converting enzyme. *J. Immunol*., **175**, 4009–4016.

Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gorman, D., Zurawski, S., Abrams, J., Menon, S.,

McClanahan, Y., De Waal-Malefyt, R. R., Bazan, F., Kastelein, R. A. & Liu, Y. J. (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol*., **3**, 673–680.

Spurrell, J. C. L., Wiehler, S., Zaheer, R. S., Sanders, S. P. & Proud, D. (2005) Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **289**, L85–L95.

Subauste, M. C., Jacoby, D. B., Richards, S. M. & Proud, D. (1995) Infection of a human respiratory epithelial cell line with rhinovirus. Induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J. Clin. Invest*., **96**, 549–557.

Tjabringa, G. S., Rabe, K. F. & Hiemstra, P. S. (2005) The human cathelicidin LL-37: a multifunctional peptide involved in infection and inflammation in the lung. *Pulm. Pharmacol. Ther*., **18**, 321–327.

Tosi, M. F., Hamedani, A., Brosovich, J. & Alpert, S. E. (1994) ICAM-1-independent, CD18-dependent adhesion between neutrophils and human airway epithelial cells exposed in vitro to ozone. *J. Immunol*., **152**, 1935–1942.

Tschumperlin, D. J. & Drazen, J. M. (2006) Chronic effects of mechanical force on airways. *Annu. Rev. Physiol*., **68**, 563–583.

van Wetering, S., Zuyderduyn, S., Ninaber, D. K., Van Sterkenburg, M. A. J. A., Rabe, K. F. & Hiemstra, P. S. (2007) Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13. *Mol. Immunol*., **44**, 803–811.

Venge, J., Lampinen, M., Hakansson, L., Rak, S. & Venge, P. (1996) Identification of IL-5 and RANTES as the major eosinophilic chemoattractants in the human lung. *J. Allergy Clin. Immunol*., **97**, 1110–1115.

Vignola, A. M., Campbell, A. M., Chanez, P., Bousquet, J., Paul-Lacoste, P., Michel, F.-B. & Godard, P. (1993) HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. *Am. Rev. Respir. Dis*., **148**, 689–694.

Wang, J. H., Trigg, C. J., Devalia, J. L., Jordan, S. & Davies, R. J. (1994) Effect of inhaled beclomethasone dipropionate on expression of proinflammatory cytokines and activated eosinophils in the bronchial epithelium of patients with mild asthma. *J. Allergy Clin. Immunol*., **94**, 1025–1034.

Wark, P. A. B., Johnston, S. L., Bucchieri, F., Powell, R., Puddicombe, S., Laza-Stanca, V., Holgate, S. T. & Davies, D. E. (2005) Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J. Exp. Med*., **201**, 937–947.

Yang, D., Biragyn, A., Hoover, D. M., Lubkowski, J. & Oppenheim, J. J. (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu. Rev. Immunol*., **22**, 181–215.

Yao, P. M., Maitre, B., Delacourt, C., Buhler, J. M., Harf, A. & Lafuma, C. (1997) Divergent regulation of 92-kDa gelatinase and TIMP-1 in HBECs in response to IL-1beta and TNF-alpha. *Am. J. Physiol. Lung Cell. Mol. Physiol*., **273**, L866–L874.

Ying, S., O'Connor, B., Ratoff, J., Meng, Q., Mallett, K., Cousins, D., Robinson, D., Zhang, G., Zhao, J., Lee, T. H. & Corrigan, C. (2005) Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J. Immunol*., **174**, 8183–8190.

Yoon, H. J., Zhu, Z., Gwaltney, J. M. J. & Elias, J. A. (1999) Rhinovirus regulation of IL-1 receptor antagonist in vivo and in vitro: a potential mechanism for symptom resolution. *J. Immunol*., **162**, 7461–7469.

Zen, K. & Parkos, C. A. (2003) Leukocyte–epithelial interactions. *Curr. Opin. Cell Biol*., **15**, 557–564.

Zen, K., Babbin, B. A., Liu, Y., Whelan, J. B., Nusrat, A. & Parkos, C. A. (2004) JAM-C is a component of desmosomes and a ligand for CD11b/CD18-mediated neutrophil transepithelial migration. *Mol. Biol. Cell*, **15**, 3926–3937.

Zheng, S., De, B. P., Choudhary, S., Comhair, S. A. A., Goggans, T., Slee, R., Williams, B. R. G., Pilewski, J., Haque, S. J. & Erzerum, S. C. (2003) Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis. *Immunity*, **18**, 619–630.

17

The Epithelium and Airway Remodelling

Stephen T. Holgate
School of Medicine, Southampton General Hospital, Southampton, UK

17.1 The epithelium and airway remodelling

Remodelling of the airway may be considered as altered structure, and as a consequence function, of the airway in response to a wide range of stimuli, most of which are associated with epithelial injury and aberrant repair. This aspect of airway disease is intimately linked to chronic airway inflammation, with both remodelling and inflammation contributing to a wide range of chronic lung disorders including asthma, COPD, bronchiectasis and cystic fibrosis. Remodelling of the lung is also central to the pathogenesis of interstitial pulmonary fibrosis and to a range of organizing pneumonias. However, it is in the field of asthma that this subject has been most widely researched and this review will therefore concentrate on asthma as an example of the disease where remodelling contributes to pathophysiology (Holgate *et al*., 2004).

It is now clear that, rather than being a single disease, asthma is a spectrum of disorders that is initiated at different stages through life by a range of environmental factors interacting with a susceptible genetic background (Wenzel, 2006). Most simply, asthma is divided into allergic or non-allergic asthma but even within each of these two broad categories there is enormous heterogeneity, both with respect to clinical and physiological manifestations of the disease, its symptoms, response to treatment and natural history (Wardlaw *et al*., 2002). Both in allergic and non-allergic asthma, Th-2 type T-lymphocyte-driven inflammation with involvement of mast cells, eosinophils, basophils and macrophages underpins the chronic inflammatory response that is so characteristic of this disease. It is also the airway inflammation against which much of the current therapy for asthma is directed, especially corticosteroids, mast cell stabilizing agents and anti-leukotrienes. Associated with the chronic inflammatory response are important interactions between the airway and the breathed environment, with pivotal roles being played by allergens, infectious agents, including viruses, bacteria and fungi, environmental tobacco smoke and a range of chemicals including

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

outdoor air pollutants (Eder *et al*., 2006). While over 80 per cent of asthma is associated with atopy (the genetic predisposition to generate IgE against common environmental allergens), over 50 per cent of the population in the developed world is atopic and yet only 7–9 per cent express this in the form of asthma (Pearce *et al*., 1999). Thus, there must be some important factors that translate the atopic phenotype into the lower airway manifestion of asthma. Antigen presentation and processing within the airway are likely to be critical processes. These include factors that determine the response of dendritic cells at the surface of the airway to inhaled allergens and other stimuli and how they communicate this to T-cells for amplifying the inflammatory response (Hammad and Lambrecht, 2006). The relationship between the adaptive immune response to environmental allergens and pathogens in relation to the subsequent airway response will be discussed later.

As in other chronic inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease and psoriasis, chronic immune and inflammatory responses are associated with a variable degree of structural change in the target tissue. In the case of asthma, the extent of inflammation, and the structural changes seen in the airway, are related to disease severity but are not dependent on either atopy or duration of symptoms (Bai and Knight, 2005). It has also been assumed that airway inflammation is fundamental to the origins of asthma but this is now being questioned with the discovery that, even early in life, the onset of asthma is associated with marked structural changes in the relative absence of airway inflammation (Barbato *et al*., 2006; Fedorov *et al*., 2005). Thus, when considering the concept of 'remodelling' of the airway in asthma one should also consider what connection this has with airway 'modelling' during fetal lung branching and morphogenesis (Bousquet *et al*., 2000). Interactions between organ morphogenesis and wound-healing responses are leading to new concepts about how chronic airway inflammation can be supported by a remodelled airway and vice versa. When considering the individual components of airway remodelling in asthma there are five features that have been identified (Figure 17.1):

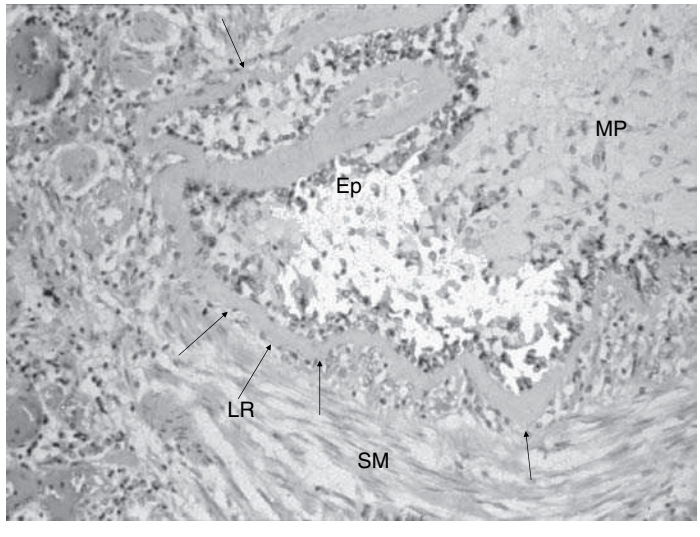


Figure 17.1 Cross-section of a large airway from a patient who died from asthma. Note the extensive damage to the airway epithelium. The arrows highlight the marked thickening and hyalinization of the lamina reticularis (LR) in asthma. SM, smooth muscle; Ep, epithelium; MP, mucous plug. (Reproduced courtesy of Holgate ST and Polosa R, *Lancet* 2006 Aug. 26, **368**(9537): 780–793)

1. epithelial damage and impaired repair
2. epithelial mucous metaplasia and submucosal gland hypertrophy and hyperplasia
3. deposition of matrix protein and proteoglycans in the lamina reticularis beneath the epithelium
4. the deposition of matrix proteins and proteoglycans in the submucosa, smooth muscle and in the airway adventitia
5. microvascular and neural network remodelling

In chronic asthma, a new theme for pathogenesis is emerging of a damaged epithelium which repairs incompletely leading to a chronic 'wound response' in the airway and the subsequent secretion of a range of growth factors that drive sub-epithelial remodelling (Davies *et al*., 2003; Knight and Holgate, 2003). In order to understand the relationship between the epithelium and these other events, it is important to break down the pathophysiological processes involved into several stages.

17.2 Epithelial injury and impaired repair

The human lung is the largest surface that is in continuous contact with the outside world and presents an estimated area of 100 m^2 that comes into contact with inspired air. Every day approximately 10 000 litres of ambient air are inhaled containing numerous potentially harmful physical, chemical and biological agents. The airway epithelium provides the interface with the breathed environment and, in this respect, forms a physical barrier against these agents to provide the first line of defence which is then complemented by mucociliary clearance. Initially thought to be pseudo-stratified, the large conducing airway are now considered to be lined by a stratified epithelium comprising ciliated epithelial cells, mucus-producing goblet cells, non-ciliated bronchiolar Clara cells and basal cells (van Winkle *et al*., 2004). The conducting airway epithelium is covered by surface liquid approximately 10 μm thick, which comprises a periciliary layer around the microvilli and an overlying mucus layer. The separation of the airway surface liquid into two layers enable the cilia to beat and subsequently move mucus onto which inhaled particles are trapped. Under normal circumstances the epithelium forms a highly regulated and impermeable barrier through the formation of tight junctions (TJs) at the apical aspect of the columnar cells. These TJs are comprised of complex proteins including Zona occludens 1–3, occludins and claudins as well as trans-membrane adhesion proteins (β-catenin, E-cadherin and JAM) that enable communication between adjacent cells (see Chapter 2) (Shin *et al*., 2006). In addition, structural integrity of the epithelium is maintained through cell–cell, and cell–extracellular matrix, interactions involving desmosomes and hemidesmosones (Roche *et al*., 1993). Disruption of the columnar epithelium will enable potentially tissue-damaging agents and infectious particles to penetrate into the airway tissue, thereby stimulating an immune and inflammatory response with subsequent tissue damage (Figure 17.1). In asthma there is both in vivo and in vitro evidence that the barrier function of the airway epithelium is impaired, which increases the susceptibility of the airway to injury (Ilowite *et al*., 1989; Knight, 2002). Using epithelial cells brushed from normal and asthmatic airway, and subsequently cultured in vitro and differentiated at an air–liquid interface (ALI), we have demonstrated that, in asthma, the epithelium is defective in its ability to form effective TJs, thereby enabling external agents to pass through the epithelium to the basal layer and beyond. Certain environmental factors, such as respiratory viruses and proteolytically active allergens, such as *DerPI*

cysteine protease, also have the capacity to disrupt tight junctions and increase epithelial permeability (Wan *et al*., 2000).

Since many of the injurious chemical, particulate and biological insults to the airway mediate their tissue-damaging effects through the generation of reactive oxygen species. The normal airway epithelium is well equipped with antioxidant enzymes, as well as active radical traps that helps maintain epithelial integrity (Rahman *et al*., 2006). However, in asthma, there is increasing evidence of defective antioxidant pathways, including deficiencies in superoxide dismutase and glutathione peroxidase. These deficiencies help explain why the asthmatic airway epithelial epithelium in vitro is more susceptible to oxidant-induced damage (Bucchieri *et al*., 2002; Comhair *et al*., 2001; Rahman *et al*., 2006).

An important question is whether, in vivo, the asthmatic airway epithelium exhibits features of chronic damage. Although 'epithelial desquamation' has been described as a pathological feature of asthma death for many years (Laitinen and Laitinen, 1994; Montefort *et al*., 1993), its significance has never really been appreciated until relatively recently (Shahana *et al*., 2006; Shebani *et al*., 2005). Bronchial biopsy studies from patients with asthma of increasing severity, not only demonstrate physical damage to the columnar cell layer, but also evidence for injury through the expression of cell stress indices, such as heat shock protein (HSP) 70 (Bertorelli *et al*., 1998), activation of the caspase enzyme cascade involved in premature programmed cell death (Bucchieri *et al*., 2002; Truong-Tran *et al*., 2002) and surface expression of epidermal growth factor receptors (EGFRs) (Hamilton *et al*., 2003, 2005; Polosa *et al*., 2002) (Figure 17.2). These markers of 'injury' are

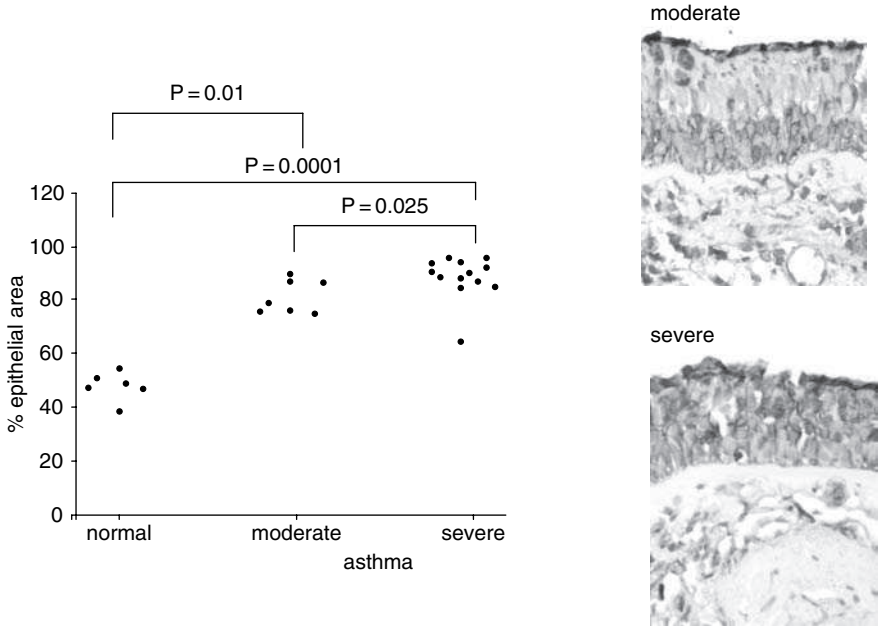


Figure 17.2 Increased expression of the epidermal growth factor receptor (EGFR) in the airway epithelium in moderate and severe childhood asthma (right) with quantification by image analysis compared to normal (left). In moderate asthma expression of EGFR is most evident in the basal cells, whereas in severe disease expression is throughout the epithelium. Note also the mucous metaplasia and the thickening of the sub-epithelial lamina reticularis. (Reproduced courtesy of Fedorov, I. *et al.*, 2005)

present even the mildest forms of asthma, but also increase in proportion to disease severity and chronicity. Almost identical changes are seen in the airway epithelium in children with early onset asthma (Fedorov *et al*., 2005), leading to the conclusion that this process is fundamental to the origins of the disease. Under normal circumstances, injury to the epithelium, whether initiated by chemical, physical, or biological agents, should stimulate pathways such as engagement of EGFRs by appropriate ligands (EGF, amphiregulin and Heparin Binding-EGF) to drive the proliferation and subsequent differentiation that are features of the primary repair response. In addition to there being evidence of increased injury and premature apoptosis in asthma, the airway epithelium is also deficient its ability to repair. This is manifested by the reduced expression of markers of cell proliferation in the nuclei of basal epithelial cells, such as Ki67 and proliferating cell nuclear antigen (PCNA), along with an impaired ability of the epithelium to restitute itself following injury as a result of overexpression of cell cycle inhibitors in the nuclei, such as $P21^{waf}$ (Fedorov *et al*., 2005; Knight and Holgate, 2003) (Figure 17.3). This leads to a 'chronic wound' scenario, or healing by 'secondary intention', that characterizes the epithelial response in chronic asthma. An abnormal epithelium is also the source of increased production of a number of cytokines and chemokines including interleukin (IL)-8, GM-CSF, RANTES, and MCP3, as well as overexpression of receptors, such as protease-activated receptor 2 (PAR2) (Knight and Holgate, 2003). Kicic and colleagues (Kicic *et al*., 2006) have reported that airway epithelial cells obtained from children with asthma and grown to confluence in vitro demonstrate increased production of prostaglandin E_2 and IL-6, and evidence of increased (rather than decreased) epithelial proliferation. Because the functional and cellular abnormalities reported in asthmatic epithelial cells are preserved with successive passage in culture, it is thought that, as found in adult asthma, these defects may well be intrinsic to the origins of the disease and not the secondary to airway wall inflammation.

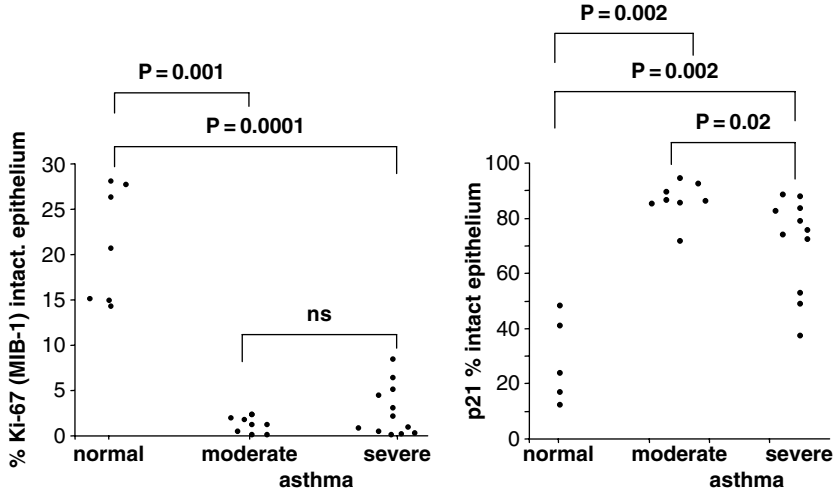


Figure 17.3 Image analysis to quantify the expression of the proliferation marker Ki67 (left) and the cell cycle inhibitor p21 (right) in the nuclei of the airway epithelium in moderate and severe childhood asthma compared to normal control. (Reproduced courtesy of Fedorov, I. *et al.*, 2005)

17.3 Changes to the epithelial laminar reticularis (basal laminar)

In both adults and children, another highly characteristic feature of asthma is the hyalinization and thickening of the laminar reticularis beneath the epithelial basement membrane (Figure 17.4). This pathological abnormality is accompanied by an increase in the number and activity of sub-epithelial myofibroblasts, with their capacity to lay down new matrix proteins at the site, including tenasin C, fibronectin and, types I, III and IV collagens (Karjalainen *et al*., 2003; Roche *et al*., 1989). The fact that these changes are present in biopsies from the airway of children with asthma soon after its inception (Figure 17.5), suggests that they start at, or soon after, the disease origin (Payne *et al*., 2003; Pohunek *et al*., 2005; Saglani *et al*., 2005). The mechanisms involved in this 'modelling' of the basal lamina are not known, although, in a range of animal models, similar changes can be induced when the epithelium is injured chronically to stimulate the release of a range of profibrotic growth factors, especially transforming growth factor (TGF)-β (Leung *et al*., 2006; Locke *et al*., 2007). The airway epithelium has the capacity to generate fibroblast growth factor (FGF)-1, FGF-2, platelet-derived growth factors, IgEs and TGF-β_2, all of which are able to interact with fibroblasts to cause their proliferation or differentiation into myofibroblasts (Zhang *et al*., 1999). Because these growth factors are overexpressed in the airway epithelium of

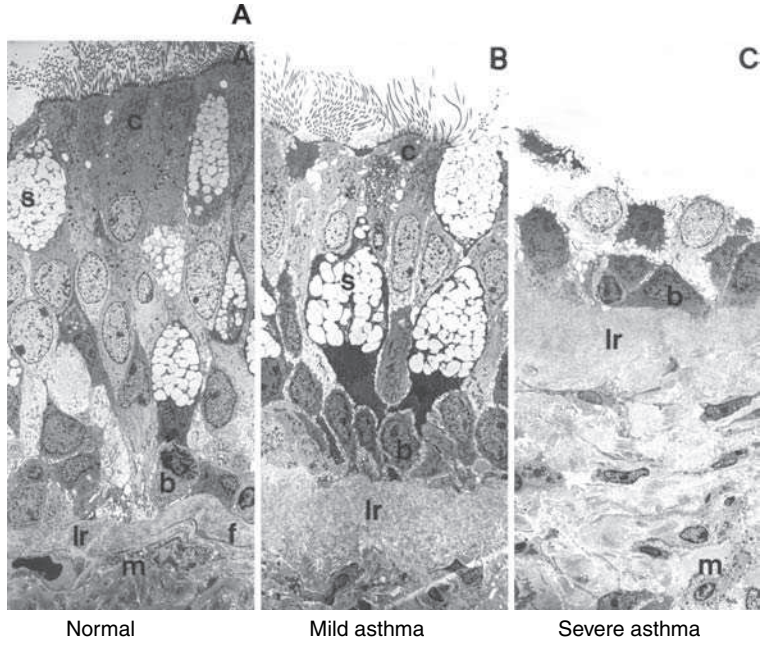


Figure 17.4 Transmission electron micrographs of normal (A), mild (B) and severe (C) asthmatic epithelium to show goblet cells (s), thickened lamina reticularis (lr) and epithelial damage characteristic of severe disease. Note the spaces between the epithelial cells evident in mild asthma. f, fibroblast; m, macrophage; b, basal epithelial cell

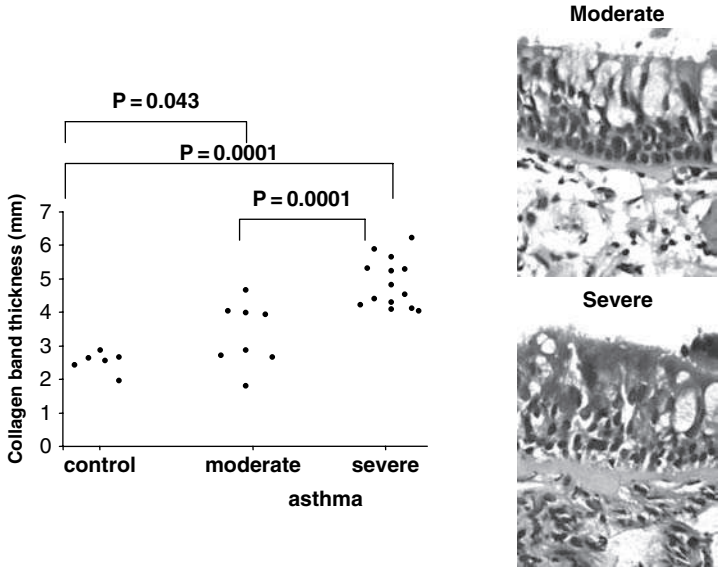


Figure 17.5 Haematoxylin and eosin stained sections of airway biopsies from children aged 7–9 with moderate and severe asthma showing the hyaline thickening of the lamina reticularis and goblet cell metaplasia (right) and the quantification of the collagen layer by image analysis when compared to normal control children (left). (Reproduced courtesy of Fedorov, I *et al.*, 2005)

patients with asthma, it has been assumed that the epithelial cell is the principal source for the profibrogenic factors that contribute to thickening of the subepithelial basal lamina. Factors that may contribute to the induction of growth factor secretion include mechanical stress imposed upon the epithelium as well as an interaction with inflammatory cells, especially eosinophils (Choe *et al*., 2003; Phipps *et al*., 2004).

Eosinophils are considered to be fundamental to the inflammatory response of asthma but despite having a major impact in reducing circulating and sputum eosinophils, the blocking anti-IL5 monoclonal antibody mepolizumab, when administered to patients with asthma, only reduced tissue eosinophils in the airway by approximately 50 per cent (Flood-Page *et al*., 2003a), possibly due to loss of IL-5 receptors from a proportion of eosinophils as they enter the airway from the circulation. While anti-IL-5 has no effect on the allergen-induced early- or late-phase reactions, bronchial hyperresponsiveness or clinical manifestations of chronic asthma, three infusions of mepolizumab over a period of 10 weeks resulted in reduced immunostaining for tenascin C, collagen III and lumican in the lamina reticularis (Flood-Page *et al*., 2003b), implying a role for eosinophils in contributing to this pathological feature of asthma (Figure 17.6). Human eosinophils are an important source of TGF-β, which has been shown to drive differentiation of sub-epithelial fibroblasts into myofibroblasts (Kay *et al*., 2004). Interestingly, thickening and hyalinization of the lamina reticularis of the epithelial basement membrane is also a characteristic of cough-variant asthma (eosinophilic bronchitis) (Brightling *et al*., 2003) indicating that, while being a marker of epithelial injury and aberrant repair, this change need not be associated with bronchial hyperresponsiveness and variable airway obstruction that characterizes asthma.

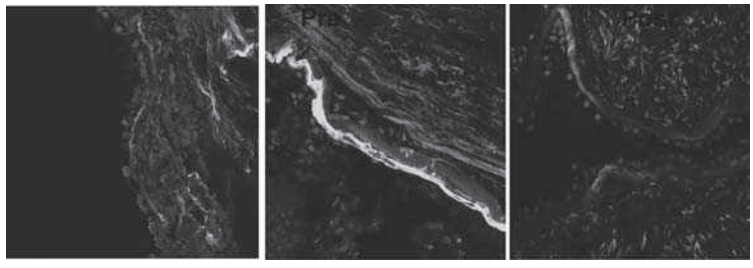


Figure 17.6 Immunoreactive tenascin in the sub-epithelial basement membrane region in normal (left), asthmatic before (middle) and 12 weeks after (right) three injections of the IL-5 blocking monoclonal antibody mepolizumab. Note the increased tenascin in asthma and its loss after anti-IL-5 treatment. (Reproduced with permission from Flood-Page, P. *et al.*, 2003b)

17.4 Submucosal smooth muscle adventitial deposition of matrix

Using high-resolution computed tomography (HRCT) there is good evidence to show that asthma chronicity and severity is associated with thickening of the airway walls (Lee *et al*., 2004b; Vignola *et al*., 2004). Pathological analysis has also shown the deposition of matrix proteins, such as collagens I and III, as well as proteoglycans in the submucosa and airway smooth muscle that may, in part, be responsible for this airway wall thickening (Pepe *et al*., 2005; Pini *et al*., 2007). Using either HRCT (Park *et al*., 1997), or an ultrasound bronchoscopic probe (Shaw *et al*., 2004), to examine airway dimensions, bronchial hyperresponsiveness has been shown to be inversely related to airway wall thickness suggesting that the latter may initially be a compensatory response to help protect the airway against repeated contraction. In the long term, however, airway wall thickening associated with deposition of matrix will lead to an irreversible component of airflow obstruction and it is this that is most commonly referred to as airway wall 'remodelling'.

The presence of activated fibroblasts in the conducting airway has recently aroused further interest in the possibility that epithelial cells themselves may undergo transdifferentiation to fibroblasts, or that primitive 'fibrosites' may be recruited from the bone marrow via the circulation into the asthmatic airway (Nihlberg *et al*., 2006). While there is some evidence for epithelial parenchymal transition in interstitial lung disease (Kim *et al*., 2006) and in animal models (Wu *et al*., 2007), evidence that this process plays a role in airway remodelling in asthma is lacking. However, repeated allergen provocation of asthmatic airway can lead to the recruitment of primitive fibrocytes into the airway and bronchoalveolar lavage fluid, with a possibility that these contribute to airway wall remodelling (Schmidt *et al*., 2003). The precise functions of matrix proteins in the remodelled asthmatic airway remain unknown, and our understanding is further hampered by not having any good methods to measure the functional significance of matrix protein deposition. Nevertheless, the relentless decline in baseline lung function that is known to occur in chronic asthma over time, and that is only partly modified by corticosteroid therapy, is thought to be in large part the result of the remodelling component of asthma (Postma and Timens, 2006; ten Brinke *et al*., 2001).

17.5 Airway smooth muscle

There is overwhelming evidence that the airway smooth muscle in asthma is highly abnormal. Not only does it exhibit abnormal contractile features but it also is a potent source of pro-inflammatory cytokines, chemokines, growth factors and other mediators (Hirst, 2003). Although there has been much debate stretched over many years about the nature of the airway smooth muscle in asthma, its increase in volume represents a combination of hypertrophy and hyperplasia. In addition, the airway smooth muscle is an important source of matrix proteins that include collagen I and a range of proteoglycans, including versican and biglycan (de Medeiros *et al*., 2005; Parameswaran *et al*., 2006). Of particular interest is the increase in proteoglycans and other matrix proteins between smooth muscle bundles in the asthmatic airway suggesting an important role in controlling their contractility and also altering the impact that this has in reducing airway luminal dimensions (Pepe *et al*., 2005; Pini *et al*., 2007). Airway smooth muscle in asthma is also able to support a chronic inflammatory response including the persistence of mast cells with their capacity to secrete mediators that could influence smooth muscle contraction and proliferative behaviour (Begueret *et al*., 2007). It is possible that what diffentiates cough variant asthma (eosinophilic bronchitis) from true asthma with variable airflow obstruction is a change in the structure–function relationships, and inflammatory cell infiltrate, in the smooth muscle compartment (Agarwal and Gupta, 2006).

Based upon an active role of the epithelium and underlying membrane in asthma pathogenesis, it is of particular interest to look for genes that may identify susceptibility to bronchial hyperresponsiveness as a sub-phenotype of asthma. One such gene, *ADAM33*, has been identified through positional cloning (Holgate *et al*., 2006; Van Eerdewegh *et al*., 2002). Mutations in this gene, have been linked not only to impaired lung function in infancy and bronchial hyperresponsiveness later in life (Haitchi *et al*., 2005; Van Eerdewegh *et al*., 2002) but also to accelerated decline in baseline lung function over time irrespective of treatment (Gosman *et al*., 2007; Jongepier *et al*., 2004; van Diemen *et al*., 2005). ADAM33 is a complex molecule comprising of 23 exons with multiple functions that not only include its metalloprotease activity but also its effect in promoting cell migration, cell fusion and changes in cell function through intercellular signalling (Holgate *et al*., 2006). At least six splice variants of ADAM33 exist, but their respective functions are unknown (Powell *et al*., 2004). One possibility is that ADAM33, in serving as a proteolytic enzyme capable of liberating growth factors from their cell-bound precursors, could be involved in smooth muscle proliferation and differentiation. A similar role has recently been described for ADAM12 in cardiac hypertrophy (Asakura *et al*., 2002; Fedak *et al*., 2006).

17.6 Vascular remodelling

Although it has been widely recognized that the microvasculature in asthma is pivotal to airway wall edema and in the recruitment of immune and inflammatory cells into the airway, it is only recently that the importance of the microvascular bed has been appreciated in relation to the remodelling response (Hashimoto *et al*., 2005). Both in children (Barbato *et al*., 2006), and in adults (Feltis *et al*., 2006; Hashimoto *et al*., 2005), the number of, and state of activation of, small blood vessels in the airway wall of asthmatics is greatly increased. Factors associated with this are being pursued, with much interest focusing on vascular

endothelial growth factor (VEGF), which is generated both by the damaged epithelium and by a number of inflammatory and structural cells in the airway wall itself (Abdel-Rahman *et al*., 2006; Bhandari *et al*., 2006; Feltis *et al*., 2006; Lee *et al*., 2004a). VEGF can cause microvascular leakage as well as drive microvascular proliferation (McDonald, 2001).

17.7 Neural networks

There is a long history of nerves being involved in asthma pathogenesis but relatively little is known about the factors that influence their control (Groneberg *et al*., 2004). The discovery of a family of nerve growth factors (NGFs, neurotrophins) produced by epithelial cells that can stimulate neural proliferation (Hazari *et al*., 2007), and interact with other elements of the airway, including mast cells (Kassel *et al*., 2001) and eosinophils (Hahn *et al*., 2006), has reinforced the idea that neural pathways are an important component of the inflammatory and remodelling responses. Circulating levels of NGF have been closely associated with asthma chronicity and severity (Bonini *et al*., 1996) and the fact that the epithelium is a major source of NGF (Bonini *et al*., 1996; Fox *et al*., 2001) raises the important issue of whether the epithelium itself helps to orchestrate the neural response. Both local and central reflex pathways have been implicated in control of smooth muscle tone, vascular permeability, mucus secretion and inflammation. However, no single set of neurotransmitters appear to dominate, and this may explain the rather disappointing results obtained with selective neuropeptide and muscarinic antagonists in asthma therapy.

17.8 Production of mucus

One feature that characterizes chronic asthma is the production of excess and altered mucus that blocks the peripheral airway and is difficult to expectorate. There is strong evidence that, in asthma, there is goblet cell metaplasia involving the conducting airway (Ordonez *et al*., 2001). The goblet cells also spread down to the more peripheral airway (Shimura *et al*., 1996) and secrete abnormally large amounts of the highly viscous mucins 5AC and 5B, which may contribute, along with DNA and eosinophil basic proteins, to the tenacious mucus that is so characteristic of this disease (Rose and Voynow, 2006). Factors that lead to goblet cell metaplasia have been extensively studied and include the activation of the EGF family of receptors on epithelial cells during repair by TGFα that is released from its membrane precursor by ADAM 17 (TACE) (Burgel and Nadel, 2004; Deshmukh *et al*., 2005). In addition, IL-4 and IL-13 also induce the production of TGFα, which, through an autocrine pathway, mediates the mucous metaplasia characteristic of Th-2 mediated inflammation in asthma (Lordan *et al*., 2002). Finally, reactive oxygen species generated by epithelial damage through activation of dual oxidase 1 (Duox1) (a homologue of glycoprotein $p91^{phox}$) stimulates TGFα cleavage from its membrane precursor to promote mucous metaplasia (Shao and Nadel, 2005). These pathways that engage growth factor release are likely to be of increasing importance in the chronic mucus production associated with severe refractory disease, especially that which involves the peripheral airway where goblet cell metaplasia has been observed almost down to the alveoli not only in asthma but also in cystic fibrosis (Burgel *et al*., 2007).

17.9 Concluding comments

While airway inflammation is undoubtedly fundamental to the pathogenesis of chronic asthma, and underlies its therapeutic response to corticosteroids, increasingly airway wall remodelling is being appreciated as an important component of the disease, particularly in patients who are refractory to anti-inflammatory therapies. Studies in children and in adults suggest that aberrant communication between the airway epithelium and underlying structures can account for a proportion of the remodelling response and that this relationship can be captured by the term 'epithelial mesenchymal trophic unit' (EMTU). The EMTU is fundamental to branching morphogenesis during lung development in the fetus, utilizing many of the growth factors that are recruited in airway wall remodelling and chronic asthma to drive airway growth and binary division (Araya *et al*., 2006; Davies *et al*., 2003). ADAM33, in addition to being a susceptibility gene in chronic asthma and bronchial hyperresponsiveness, is also expressed preferentially in primitive mesenchymal cells involved in human lung morphogenesis, suggesting that this molecule has dual functions both in lung development, and in chronic asthma and its progression over time. Recognizing that the epithelium is chronically damaged in asthma, with defective formation of tight junctions, new therapeutics could be targeted to this aspect of the disease with the ability to return the epithelium to normal function and restore its barrier functions against environmental insults. Discovery that epidermal growth factor (EGF) and keratinocyte growth factor (KGF) can both restore epithelial integrity in asthmatic cultures and induce the normal formation of tight junctions provides one example of how therapies influencing this part of the asthma process may be beneficial beyond the use of anti-inflammatory and immune modulating treatments. The successful use of EGF in the treatment of corticosteroid refractory ulcerative colitis (Dieckgraefe *et al*., 2006; Sinha *et al*., 2003) and the use of KGF in preventing mucositis in patients undergoing chemotherapy for cancer and marrow transplantation (Adis International Ltd, 2004) again supports the view that increasing the resistance of the airway epithelium against the environment is a feasible way of treating asthma which does not rely on suppressing inflammation.

References

Adis International Ltd. (2004) Palifermin: AMJ 9701, KGF-Amgen, recombinant human keratinocyte growth factor, rHu-KGF, *Drugs R.D*., **5**, 351–354.

Abdel-Rahman, A. M., el Sahrigy, S. A., & Bakr, S. I. (2006) A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack, *Chest*, **129**, 266–271.

Agarwal, R. & Gupta, D. (2006) Eosinophilic bronchitis and asthma with sputum eosinophilia are different entities, *J. Allergy Clin. Immunol*., **118**, 969–970.

Araya, J., Cambier, S., Morris, A., Finkbeiner, W., & Nishimura, S. L. (2006) Integrin-mediated transforming growth factor-beta activation regulates homeostasis of the pulmonary epithelial-mesenchymal trophic unit, *Am. J. Pathol*., **169**, 405–415.

Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H., Asanuma, H., Sanada, S., Matsumura, Y., Takeda, H., Beppu, S., Tada, M., Hori, M., & Higashiyama, S. (2002) Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy, *Nat. Med*., **8**, 35–40.

Bai, T. R. & Knight, D. A. (2005) Structural changes in the airways in asthma: observations and consequences, *Clin. Sci. (Lond.)*, **108**, 463–477.

Barbato, A., Turato, G., Baraldo, S., Bazzan, E., Calabrese, F., Panizzolo, C., Zanin, M. E., Zuin, R., Maestrelli, P., Fabbri, L. M., & Saetta, M. (2006) Epithelial damage and angiogenesis in the airways of children with asthma, *Am. J. Respir. Crit. Care Med*., **174**, 975–981.

Begueret, H., Berger, P., Vernejoux, J. M., Dubuisson, L., Marthan, R., & Tunon-de-Lara, J. M. (2007) Inflammation of bronchial smooth muscle in allergic asthma, *Thorax*, **62**, 8–15.

Bertorelli, G., Bocchino, V., Zhuo, X., Chetta, A., Del Donno, M., Foresi, A., Testi, R., & Olivieri, D. (1998) Heat shock protein 70 upregulation is related to HLA-DR expression in bronchial asthma. Effects of inhaled glucocorticoids, *Clin. Exp. Allergy*, **28**, 551–560.

Bhandari, V., Choo-Wing, R., Chapoval, S. P., Lee, C. G., Tang, C., Kim, Y. K., Ma, B., Baluk, P., Lin, M. I., McDonald, D. M., Homer, R. J., Sessa, W. C., & Elias, J. A. (2006) Essential role of nitric oxide in VEGF-induced, asthma-like angiogenic, inflammatory, mucus, and physiologic responses in the lung, *Proc. Natl Acad. Sci. U.S.A*., **103**, 11021–11026.

Bonini, S., Lambiase, A., Bonini, S., Angelucci, F., Magrini, L., Manni, L., & Aloe, L. (1996) Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma, *Proc. Natl Acad. Sci. U.S.A*., **93**, 10955–10960.

Bousquet, J., Yssel, H., & Vignola, A. M. (2000) Is allergic asthma associated with delayed fetal maturation or the persistence of conserved fetal genes?, *Allergy*, **55**, 1194–1197.

Brightling, C. E., Symon, F. A., Birring, S. S., Bradding, P., Wardlaw, A. J., & Pavord, I. D. (2003) Comparison of airway immunopathology of eosinophilic bronchitis and asthma, *Thorax*, **58**, 528–532.

Bucchieri, F., Puddicombe, S. M., Lordan, J. L., Richter, A., Buchanan, D., Wilson, S. J., Howarth, P. H., Djukanovic, R., Holgate, S. T., & Davies, D. E. (2002) Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis., *Am. J. Respir. Cell Mol. Biol*., **27**, 179–185.

Burgel, P. R. & Nadel, J. A. (2004) Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium, *Thorax*, **59**, 992–996.

Burgel, P. R., Montani, D., Danel, C., Dusser, D., & Nadel, J. A. (2007) A morphometric study of mucins and small airway plugging in cystic fibrosis, *Thorax*, **62**, 153–161.

Choe, M. M., Sporn, P. H., & Swartz, M. A. (2003) An in vitro airway wall model of remodeling, *Am. J. Physiol. Lung Cell Mol. Physiol*., **285**, L427–L433.

Comhair, S. A., Bhathena, P. R., Farver, C., Thunnissen, F. B., & Erzurum, S. C. (2001) Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells, *FASEB J*., **15**, 70–78.

Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M., & Holgate, S. T. (2003) Airway remodelling in asthma – new insights., *J. Allergy Clin. Immunol*., **111**, 215–225.

de Medeiros, M. M., da Silva, L. F., dos Santos, M. A., Fernezlian, S., Schrumpf, J. A., Roughley, P., Hiemstra, P. S., Saldiva, P. H., Mauad, T., & Dolhnikoff, M. (2005) Airway proteoglycans are differentially altered in fatal asthma, *J. Pathol*., **207**, 102–110.

Deshmukh, H. S., Case, L. M., Wesselkamper, S. C., Borchers, M. T., Martin, L. D., Shertzer, H. G., Nadel, J. A., & Leikauf, G. D. (2005) Metalloproteinases mediate mucin 5AC expression by epidermal growth factor receptor activation, *Am. J. Respir. Crit. Care Med*., **171**, 305–314.

Dieckgraefe, B. K., Korzenik, J. R., & Anant, S. (2006) Growth factors as treatment options for intestinal inflammation, *Ann. N.Y. Acad. Sci*., **1072**, 300–306.

Eder, W., Ege, M. J., & von Mutius, E. (2006) The asthma epidemic, *N. Engl. J. Med*., **355**, 2226–2235.

Fedak, P. W., Moravec, C. S., McCarthy, P. M., Altamentova, S. M., Wong, A. P., Skrtic, M., Verma, S., Weisel, R. D., & Li, R. K. (2006) Altered expression of disintegrin metalloproteinases and their inhibitor in human dilated cardiomyopathy, *Circulation*, **113**, 238–245.

Fedorov, I. A., Wilson, S. J., Davies, D. E., & Holgate, S. T. (2005) Epithelial stress and structural remodelling in childhood asthma, *Thorax*, **60**, 389–394.

Feltis, B. N., Wignarajah, D., Zheng, L., Ward, C., Reid, D., Harding, R., & Walters, E. H. (2006) Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma, *Am. J. Respir. Crit. Care Med*., **173**, 1201–1207.

Flood-Page, P. T., Menzies-Gow, A. N., Kay, A. B., & Robinson, D. S. (2003a) Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway, *Am. J. Respir. Crit. Care Med*., **167**, 199–204.

Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M. S., Barnes, N., Robinson, D., & Kay, A. B. (2003b) Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics, *J. Clin. Invest*., **112**, 1029–1036.

Fox, A. J., Patel, H. J., Barnes, P. J., & Belvisi, M. G. (2001) Release of nerve growth factor by human pulmonary epithelial cells: role in airway inflammatory diseases, *Eur. J. Pharmacol*., **424**, 159–162.

Gosman, M. M., Boezen, H. M., van Diemen, C. C., Snoeck-Stroband, J. B., Lapperre, T. S., Hiemstra, P. S., Ten Hacken, N. H., Stolk, J., & Postma, D. S. (2007) A disintegrin and metalloproteinase 33 and chronic obstructive pulmonary disease pathophysiology, *Thorax*, **62**, 242–247.

Groneberg, D. A., Quarcoo, D., Frossard, N., & Fischer, A. (2004) Neurogenic mechanisms in bronchial inflammatory diseases, *Allergy*, **59**, 1139–1152.

Hahn, C., Islamian, A. P., Renz, H., & Nockher, W. A. (2006) Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation, *J. Allergy Clin. Immunol*., **117**, 787–794.

Haitchi, H. M., Powell, R. M., Shaw, T. J., Howarth, P. H., Wilson, S. J., Wilson, D. I., Holgate, S. T., & Davies, D. E. (2005) ADAM33 Expression in asthmatic airways and human embryonic lungs, *Am. J. Respir. Crit. Care Med*., **171**, 958–965.

Hamilton, L. M., Torres-Lozano, C., Puddicombe, S. M., Richter, A., Kimber, I., Dearman, R. J., Vrugt, B., Aalbers, R., Holgate, ST., Djukanovic, R., Wilson, S. J., & Davies, D. E. (2003) The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma, *Clin. Exp. Allergy*, **33**, 233–240.

Hamilton, L. M., Puddicombe, S. M., Dearman, R. J., Kimber, I., Sandstrom, T., Wallin, A., Howarth, P. H., Holgate, S. T., Wilson, S. J., & Davies, D. E. (2005) Altered protein tyrosine phosphorylation in asthmatic bronchial epithelium, *Eur. Respir. J*., **25**, 978–985.

Hammad, H. & Lambrecht, B. N. (2006) Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation, *J. Allergy Clin. Immunol*., **118**, 331–336.

Hashimoto, M., Tanaka, H., & Abe, S. (2005) Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD, *Chest*, **127**, 965–972.

Hazari, M. S., Pan, J. H., & Myers, A. C. (2007) Nerve growth factor acutely potentiates synaptic transmission in vitro and induces dendritic growth in vivo on adult neurons in airway parasympathetic ganglia, *Am. J. Physiol. Lung Cell Mol. Physiol*., **292**, L992–L1001.

Hirst, S. J. (2003) Regulation of airway smooth muscle cell immunomodulatory function: role in asthma, *Respir. Physiol. Neurobiol*., **137**, 309–326.

Holgate, S. T., Holloway, J., Wilson, S., Bucchieri, F., Puddicombe, S., & Davies, D. E. (2004) Epithelial-mesenchymal communication in the pathogenesis of chronic asthma, *Proc. Am. Thorac. Soc*., **1**, 93–98.

Holgate, S. T., Yang, Y., Haitchi, H. M., Powell, R. M., Holloway, J. W., Yoshisue, H., Pang, Y. Y., Cakebread, J., & Davies, D. E. (2006) The genetics of asthma: ADAM33 as an example of a susceptibility gene, *Proc. Am. Thorac. Soc*., **3**, 440–443.

Ilowite, J. S., Bennett, W. D., Sheetz, M. S., Groth, M. L., & Nierman, D. M. (1989) Permeability of the bronchial mucosa to 99mTc-DTPA in asthma, *Am. Rev. Respir. Dis*., **139**, 1139–1143.

Jongepier, H., Boezen, H. M., Dijkstra, A., Howard, T. D., Vonk, J. M., Koppelman, G. H., Zheng, S. L., Meyers, D. A., Bleecker, E. R., & Postma, D. S. (2004) Polymorphisms of the ADAM33 gene are associated with accelerated lung function decline in asthma, *Clin. Exp. Allergy*, **34**, 757–760.

Karjalainen, E. M., Lindqvist, A., Laitinen, L. A., Kava, T., Altraja, A., Halme, M., & Laitinen, A. (2003) Airway inflammation and basement membrane tenascin in newly diagnosed atopic and nonatopic asthma, *Respir. Med*., **97**, 1045–1051.

Kassel, O., de Blay, F., Duvernelle, C., Olgart, C., Israel-Biet, D., Krieger, P., Moreau, L., Muller, C., Pauli, G., & Frossard, N. (2001) Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated inhalation of allergen at low-dose, *Clin. Exp. Allergy*, **31**, 1432–1440.

Kay, A. B., Phipps, S., & Robinson, D. S. (2004) A role for eosinophils in airway remodelling in asthma, *Trends Immunol*., **25**, 477–482.

Kicic, A., Sutanto, E. N., Stevens, P. T., Knight, D. A., & Stick, S. M. (2006) Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma, *Am. J. Respir. Crit. Care Med*., **174**, 1110–1118.

Kim, K. K., Kugler, M. C., Wolters, P. J., Robillard, L., Galvez, M. G., Brumwell, A. N., Sheppard, D., & Chapman, H. A. (2006) Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix, *Proc. Natl Acad. Sci. U.S.A*., **103**, 13180–13185.

Knight, D. (2002) Increased permeability of asthmatic epithelial cells to pollutants. Does this mean that they are intrinsically abnormal?, *Clin. Exp. Allergy*, **32**, 1263–1265.

Knight, D. A. & Holgate, S. T. (2003) The airway epithelium: structural and functional properties in health and disease, *Respirology*, **8**, 432–446.

Laitinen, A. & Laitinen, L. A. (1994) Airway morphology: epithelium/basement membrane, *Am. J. Respir. Crit. Care Med*., **150**, S14–S17.

Lee, C. G., Link, H., Baluk, P., Homer, R. J., Chapoval, S., Bhandari, V., Kang, M. J., Cohn, L., Kim, Y. K., McDonald, D. M., & Elias, J. A. (2004a) Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung, *Nat. Med*., **10**, 1095–1103.

Lee, Y. M., Park, J. S., Hwang, J. H., Park, S. W., Uh, S. T., Kim, Y. H., & Park, C. S. (2004b) High-resolution CT findings in patients with near-fatal asthma: comparison of patients with mild-to-severe asthma and normal control subjects and changes in airway abnormalities following steroid treatment, *Chest*, **126**, 1840–1848.

Leung, S. Y., Niimi, A., Noble, A., Oates, T., Williams, A. S., Medicherla, S., Protter, A. A., & Chung, K. F. (2006) Effect of transforming growth factor-beta receptor I kinase inhibitor 2,4-disubstituted pteridine (SD-208) in chronic allergic airway inflammation and remodeling, *J. Pharmacol. Exp. Ther*., **319**, 586–594.

Locke, N. R., Royce, S. G., Wainewright, J. S., Samuel, C. S., & Tang, M. L. (2007) Comparison of airway remodeling in acute, subacute and chronic models of allergic airways disease, *Am. J. Respir. Cell Mol. Biol*., **36**, 625–632.

Lordan, J. L., Bucchieri F., Richter, A., Konstantinidis, A. K., Holloway, J. W., Puddicombe, S. M., Buchanan, D. R., Wilson, S. J., Djukanovic, R., Holgate, S. T., & Davies, D. E. (2002) Co-operative effects of Th-2 cytokines and allergen on normal and asthmatic bronchial epithelial cells., *J. Immunol*., **169**, 407–414.

McDonald, D. M. (2001) Angiogenesis and remodeling of airway vasculature in chronic inflammation, *Am. J. Respir. Crit. Care Med*., **164**, S39–S45.

Montefort, S., Djukanovic, R., Holgate, S. T., & Roche, W. R. (1993) Ciliated cell damage in the bronchial epithelium of asthmatics and non- asthmatics, *Clin. Exp. Allergy*, **23**, 185–189.

Nihlberg, K., Larsen, K., Hultgardh-Nilsson, A., Malmstrom, A., Bjermer, L., & Westergren-Thorsson, G. (2006) Tissue fibrocytes in patients with mild asthma: a possible link to thickness of reticular basement membrane?, *Respir. Res*., **7**, 50.

Ordonez, C. L., Khashayar, R., Wong, H. H., Ferrando, R., Wu, R., Hyde, D. M., Hotchkiss, J. A., Zhang, Y., Novikov, A., Dolganov, G., & Fahy, J. V. (2001) Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression, *Am. J. Respir. Crit. Care Med*., **163**, 517–523.

Parameswaran, K., Willems-Widyastuti, A., Alagappan, V. K., Radford, K., Kranenburg, A. R., & Sharma, H. S. (2006) Role of extracellular matrix and its regulators in human airway smooth muscle biology, *Cell Biochem. Biophys*., **44**, 139–146.

Park, J. W., Hong, Y. K., Kim, C. W., Kim, D. K., Choe, K. O., & Hong, C. S. (1997) High-resolution computed tomography in patients with bronchial asthma: correlation with clinical features, pulmonary functions and bronchial hyperresponsiveness, *J. Investig. Allergol. Clin. Immunol*., **7**, 186–192.

Payne, D. N., Rogers, A. V., Adelroth, E., Bandi, V., Guntupalli, K. K., Bush, A., & Jeffery, P. K. (2003) Early thickening of the reticular basement membrane in children with difficult asthma, *Am. J. Respir. Crit. Care Med*., **167**, 78–82.

Pearce, N., Pekkanen, J., & Beasley, R. (1999) How much asthma is really attributable to atopy?, *Thorax*, **54**, 268–272.

Pepe, C., Foley, S., Shannon, J., Lemiere, C., Olivenstein, R., Ernst, P., Ludwig, M. S., Martin, J. G., & Hamid, Q. (2005) Differences in airway remodeling between subjects with severe and moderate asthma, *J. Allergy Clin. Immunol*., **116**, 544–549.

Phipps, S., Benyahia, F., Ou, T. T., Barkans, J., Robinson, D. S., & Kay, A. B. (2004) Acute allergen-induced airway remodeling in atopic asthma, *Am. J. Respir. Cell Mol. Biol*., **31**, 626–632.

Pini, L., Hamid, Q., Shannon, J., Lemelin, L., Olivenstein, R., Ernst, P., Lemiere, C., Martin, J. G., & Ludwig, M. S. (2007) Differences in proteoglycan deposition in the airways of moderate and severe asthmatics, *Eur. Respir. J*., **29**, 71–77.

Pohunek, P., Warner, J. O., Turzikova, J., Kudrmann, J., & Roche, W. R. (2005) Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma, *Pediatr. Allergy Immunol*., **16**, 43–51.

Polosa, R., Puddicombe, S. M., Krishna, M. T., Tuck, A. B., Howarth, P. H., Holgate, S. T., & Davies, D. E. (2002) Expression of c-erbB receptors and ligands in the bronchial epithelium of asthmatic subjects, *J. Allergy Clin. Immunol*., **109**, 75–81.

Postma, D. S. & Timens, W. (2006) Remodeling in asthma and chronic obstructive pulmonary disease, *Proc. Am. Thorac. Soc*., **3**, 434–439.

Powell, R. M., Wicks, J., Holloway, J. W., Holgate, S. T., & Davies, D. E. (2004) The splicing and fate of ADAM33 transcripts in primary human airways fibroblasts, *Am. J. Respir. Cell Mol. Biol*., **31**, 13–21.

Rahman, I., Biswas, S. K., & Kode, A. (2006) Oxidant and antioxidant balance in the airways and airway diseases, *Eur. J. Pharmacol*., **533**, 222–239.

Roche, W. R., Beasley, R., Williams, J. H., & Holgate, S. T. (1989) Subepithelial fibrosis in the bronchi of asthmatics, *Lancet*, **1**, 520–524.

Roche, W. R., Montefort, S., Baker, J., & Holgate, S. T. (1993) Cell adhesion molecules and the bronchial epithelium, *Am. Rev. Respir. Dis*., **148**, S79–S82.

Rose, M. C. & Voynow, J. A. (2006) Respiratory tract mucin genes and mucin glycoproteins in health and disease, *Physiol. Rev*., **86**, 245–278.

Saglani, S., Malmstrom, K., Pelkonen, A. S., Malmberg, L. P., Lindahl, H., Kajosaari, M., Turpeinen, M., Rogers, A. V., Payne, D. N., Bush, A., Haahtela, T., Makela, M. J., & Jeffery, P. K. (2005) Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction, *Am. J. Respir. Crit. Care Med*., **171**, 722–727.

Schmidt, M., Sun, G., Stacey, M. A., Mori, L., & Mattoli, S. (2003) Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma, *J. Immunol*., **171**, 380–389.

Shahana, S., Jaunmuktane, Z., Asplund, M. S., & Roomans, G. M. (2006) Ultrastructural investigation of epithelial damage in asthmatic and non-asthmatic nasal polyps, *Respir. Med*., **100**, 2018–2028.

Shao, M. X. & Nadel, J. A. (2005) Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells, *Proc. Natl Acad. Sci. U.S.A*., **102**, 767–772.

Shaw, T. J., Wakely, S. L., Peebles, C. R., Mehta, R. L., Turner, J. M., Wilson, S. J., & Howarth, P. H. (2004) Endobronchial ultrasound to assess airway wall thickening: validation in vitro and in vivo, *Eur. Respir. J*., **23**, 813–817.

Shebani, E., Shahana, S., Janson, C., & Roomans, G. M. (2005) Attachment of columnar airway epithelial cells in asthma, *Tissue Cell*, **37**, 145–152.

Shimura, S., Andoh, Y., Haraguchi, M., & Shirato, K. (1996) Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma, *Eur. Respir. J.*, **9**, 1395–1401.

Shin, K., Fogg, V. C., & Margolis, B. (2006) Tight junctions and cell polarity, *Annu. Rev. Cell Dev. Biol.*, **22**, 207–235.

Sinha, A., Nightingale, J., West, K. P., Berlanga-Acosta, J., & Playford, R. J. (2003) Epidermal growth factor enemas with oral mesalamine for mild-to-moderate left-sided ulcerative colitis or proctitis, *N. Engl. J. Med.*, **349**, 350–357.

ten Brinke, A., Zwinderman, A. H., Sterk, P. J., Rabe, K. F., & Bel, E. H. (2001) Factors associated with persistent airflow limitation in severe asthma, *Am. J. Respir. Crit. Care Med.*, **164**, 744–748.

Truong-Tran, A. Q., Ruffin, R. E., Foster, P. S., Koskinen, A. M., Coyle, P., Philcox, J. C., Rofe, A. M., & Zalewski, P. D. (2002) Altered zinc homeostasis and caspase-3 activity in murine allergic airway inflammation, *Am. J. Respir. Cell Mol. Biol.*, **27**, 286–296.

van Diemen, C. C., Postma, D. S., Vonk, J. M., Bruinenberg, M., Schouten, J. P., & Boezen, H. M. (2005) A disintegrin a metalloprotease 33 polymorphisms and lung function decline in the general population, *Am. J. Respir. Crit. Care Med.*, **172**, 329–333.

Van Eerdewegh, P., Little, R. D., Dupuis, J., Del Mastro, R. G., Falls, K., Simon, J., Torrey, D., Pandit, S., McKenny, J., Brausnschweiger, K., Walsh, A., Lui, Z., Hayward, B., Folz, C., Manning, S. P., Bawa, A., Saracino, L., Thackston, M., Benchekroun, Y., Capparell, N., Wang, M., Adair, R., Reng, Y., Dubois, J., Fitzgerald, M. G., Huang, H., Gibson, R., Allen, K. M., Pedan, A., Danzig, M. R., Umland, S. P., Egan, R. W., Cuss, F. M., Rorke, S., Clough, J. B., Holloway, J. W., Holgate, S. T., & Keith, T. P. (2002) Association of the ADAM-33 gene with asthma and bronchial hyper-responsiveness, *Nature*, **418**, 426–430.

van Winkle, L. S., Fanucchi, M. V., Miller, L. A., Baker, G. L., Gershwin, L. J., Schelegle, E. S., Hyde, D. M., Evans, M. J., & Plopper, C. G. (2004) Epithelial cell distribution and abundance in rhesus monkey airways during postnatal lung growth and development, *J. Appl. Physiol.*, **97**, 2355–2363.

Vignola, A. M., Paganin, F., Capieu, L., Scichilone, N., Bellia, M., Maakel, L., Bellia, V., Godard, P., Bousquet, J., & Chanez, P. (2004) Airway remodelling assessed by sputum and high-resolution computed tomography in asthma and COPD, *Eur. Respir. J.*, **24**, 910–917.

Wan, H., Winton, H. L., Soeller, C., Gruenert, D. C., Thompson, P. J., Cannell, M. B., Stewart, G. A., Garrod, D. R., & Robinson, C. (2000) Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1, *Clin. Exp. Allergy*, **30**, 685–698.

Wardlaw, A. J., Brightling, C. E., Green, R., Woltmann, G., Bradding, P., & Pavord, I. D. (2002) New insights into the relationship between airway inflammation and asthma, *Clin. Sci. (Lond.)*, **103**, 201–211.

Wenzel, S. E. (2006) Asthma: defining of the persistent adult phenotypes, *Lancet* **368**, 804–813.

Wu, Z., Yang, L., Cai, L., Zhang, M., Cheng, X., Yang, X., & Xu, J. (2007) Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse, *Respir. Res.*, **8**, 1.

Zhang, S., Smartt, H., Holgate, S. T., & Roche, W. R. (1999) Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma, *Lab. Invest.*, **79**, 395–405.

18

Modulation of Epithelial Cell Function by Glucocorticoids: Anti-inflammatory and Other Effects

Robert Newton, Manminder Kaur, Elizabeth M. King, Wei Gong, Donna M. Slater and **Neil S. Holden**
Department of Cell Biology & Anatomy, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

18.1 Introduction

Glucocorticoids (corticosteroids, glucocorticosteroids or often just steroids) are among the most effective and widely used anti-inflammatory medications for the treatment of inflammatory airway diseases including asthma, allergic rhinitis, chronic obstructive pulmonary disease, fibrosis, and others (Rhen and Cidlowski, 2005; Barnes, 2006). In the context of airway diseases, the airway epithelium is a key source of inflammatory mediators and is now recognized for its important immuno-modulatory role (Mills *et al*., 1999). Furthermore, the airway epithelial cell is the first cell type to receive external insults, which may include noxious chemicals, cold air, dust, carbon and other particulates, viruses, bacteria and their products (Mills *et al*., 1999; Proud and Chow, 2006). In addition, cytokines, such as interleukin (IL) 1β or tumour necrosis factor (TNF)α, which are released from activated macrophages, lead to epithelial cell activation. This position at the interface with the external environment, combined with its ability to produce inflammatory mediators, makes the airway epithelium a critical target for inhaled therapeutic agents. In this context, glucocorticoids are, without doubt, the most significant anti-inflammatory agents that are delivered to the airway epithelium and these act to down-regulate, or repress, the expression of multiple inflammatory mediators that are produced in response to inflammatory insults. Mechanisms for this effect will be discussed in detail in the main bulk of this chapter and, in addition, various other aspects of glucocorticoid action on the airway epithelium shall be considered.

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

18.2 Expression of inflammatory genes by epithelial cells is reduced by glucocorticoids

Following inflammatory insult, for example by the pro-inflammatory cytokines IL-1β or TNFα, epithelial cells are stimulated to produce multiple cytokines (e.g. IL-1β, TNFα, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF)), chemokines (e.g. IL-8, regulated on activation, normal T-cell expressed and secreted (RANTES), monocyte chemoattractant peptide (MCP)-1), inflammatory enzymes (e.g. cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS)), adhesion molecules (intercellular adhesion molecule (ICAM)-1), as well as a variety of other mediators, including lipids and host defence molecules and products (see Chapter 16). However, in the presence of glucocorticoids, the expression, or production, of many of these mediators, although possibly not host defence proteins, is strongly inhibited (Schleimer, 2004). Thus, in pulmonary epithelial cells, the expression of classic inflammatory cytokines and chemokines including IL-6, IL-8 and GM-CSF are strongly induced by stimuli such as TNFα or IL-1β, and these responses are repressed by glucocorticoids such as dexamethasone (Levine *et al*., 1993; Adkins *et al*., 1998; Kwon *et al*., 1994). Furthermore, such effects are also evident in the asthmatic epithelium in vivo and in primary human airway epithelial cells (HAEC) treated with soluble factors from *Staphyloccus aureus* (Fragaki *et al*., 2006; Wang *et al*., 1994). Likewise, the chemoattractants MCP-1, MCP-4, eotaxin and RANTES are induced in pulmonary epithelial cells, including type II alveolar and bronchial epithelial cells, and are also repressed by dexamethasone (Paine *et al*., 1993; Stellato *et al*., 1995, 1999; Lilly *et al*., 1997). The repressive effect of glucocorticoids extends to the expression of inflammatory proteins, such as cyclooxygenase (COX) 2 and inducible nitric oxide synthase (iNOS) (Mitchell *et al*., 1994; Kleinert *et al*., 1996; Newton *et al*., 1998a), as well as adhesion molecules, such as ICAM-1 and vascular cell adhesion molecule (VACM)-1 (van de Stolpe *et al*., 1993; Atsuta *et al*., 1999), which are both expressed on airway epithelial cells and collectively contribute to the inflamed state.

18.3 Modulation of transcription by GR at simple GREs

Despite the cloning of the glucocorticoid receptor in 1985 (Hollenberg *et al*., 1985), the mechanisms by which inflammatory gene expression is repressed by glucocorticoids still require considerable elucidation. Certainly, the realization that GR is modular in structure was instrumental to defining the core paradigm of glucocorticoid action (Giguere *et al*., 1986). Thus, binding of glucocorticoid to the ligand binding domain (LBD) promotes nuclear translocation of GR as a result of dissociation from heat shock protein (hsp) 90. Historically, this effect was thought to occur in the cytoplasm, but more recent studies suggest that dissociation from hsp90 occurs in the nucleus and that it is the ligand-dependent loss and recruitment of other associated proteins that is important for translocation to the nucleus (Pratt *et al*., 2004; Davies *et al*., 2002) (Figure 18.1). In the nucleus, GR can bind and dimerize, via the DNA binding domain (DBD), to imperfect DNA palindromes (consensus: 5′ - GGT ACA NNN TGT TCT - 3′) (Luisi *et al*., 1991), known as glucocorticoid response elements (GREs). This allows transcriptional activation (transactivation) by virtue of the activation functions AF1 and AF2 that are present in GR (Rhen

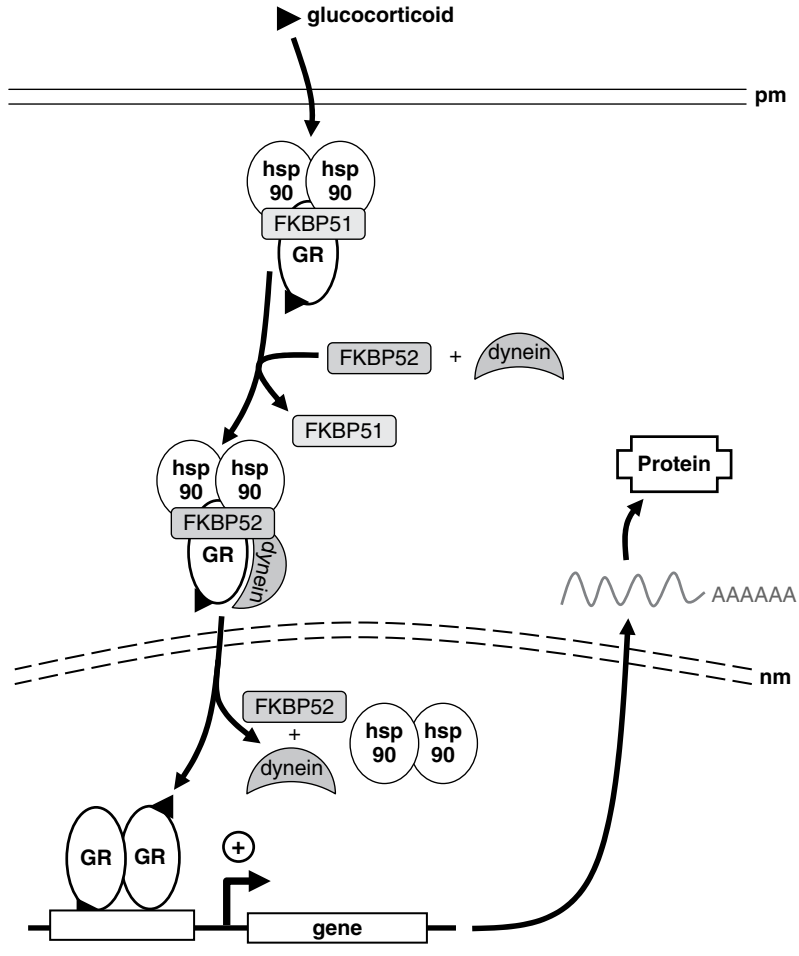


Figure 18.1 Schematic showing the activation of a simple GRE following the binding of glucocorticoid ligand to GR. The schematic depicts glucocorticoid passing through the plasma membrane (pm) where it binds to the glucocorticoid receptor (GR) causing exchange of FKBP51 for FKBP52 and binding of dynein. The ligand-bound GR complex then translocates across the nuclear membrane (nm) and into the nucleus where hsp90, FKBP52 and dynein dissociate. Binding of ligand-bound GR and dimerization on a simple GRE is depicted. This allows transcriptional activation of target genes to produce mRNA and then protein. Adapted from *J Bio Chem* **277**: 4597–4600

and Cidlowski, 2005). Thus, genes including tyrosine amino transferase (TAT), tryptophan oxygenase and phosphoenol pyruvate carboxykinase (PEPCK) were shown to have positive glucocorticoid responsive sites in their promoter regions, and this appeared to account for inducibility by glucocorticoids (Newton, 2000; Schacke *et al*., 2002). While this scheme of GR acting at simple positive GREs was thought to account for many of the metabolic effects of glucocorticoids (Schacke *et al*., 2002) (Figure 18.2), anti-inflammatory properties attributed to transactivation were less obvious. For example, lipocortin I (annexin I)

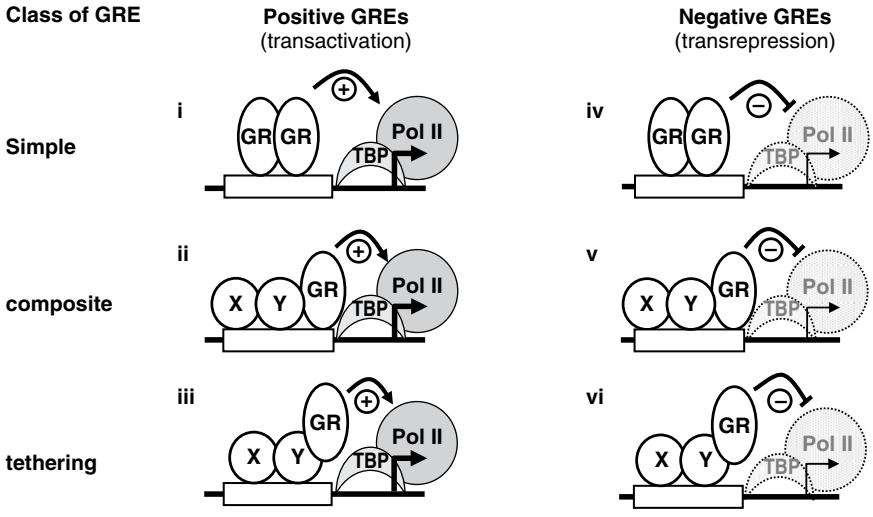


Figure 18.2 Different types of transcriptional response elicited by the glucocorticoid receptor (GR) in the nucleus. GR may be recruited to transcriptional promoter regions by binding DNA either as a homodimer in the context of simple GRE sites (i, iv), or as a composite site (ii, v) that binds DNA in conjunction with other transcription factors, or finally via interaction with another transcription factor, but without actually contacting the DNA itself (i.e. tethering) (iii, vi). In each scenario there may be positive or negative effects on transcription and the GRE site is accordingly described as a positive or negative GRE. The nature of the response, either positive of negative, elicited by GR needs to be communicated to the basal transcriptional machinery, which is here represented as TATA binding protein (TBP) and RNA polymerase II (Pol II)

is a glucocorticoid-inducible protein that represses phospholipase (PL) A_2 activity in a variety of cells, including the pulmonary epithelium (Flower and Rothwell, 1994). Likewise the induction of secretory leukocyte protease inhibitor (SLPI) by glucocorticoids in airway epithelial cells may be of anti-inflammatory benefit (Abbinante-Nissen *et al*., 1995). However, such effects do not explain the ability of glucocorticoids to reduce the expression of pro-inflammatory genes and this is usually attributed to other mechanisms (Barnes, 2006; Rhen and Cidlowski, 2005).

Analogy to the positive GRE led to speculation regarding the existence of simple negative GRE sites (nGREs) by which glucocorticoids could bind to DNA and down-regulate inflammatory gene transcription (Figure 18.2). Indeed simple nGRE sites were proposed in the transcriptional promoter regions of a number of genes including the pro-opiomelanocortin (POMC) gene, which is responsible for adrenocorticotropic hormone (ACTH) expression, the prolactin gene and the osteocalcin gene (Schacke *et al*., 2002; Newton, 2000). In these cases, the nGRE site corresponded poorly to a consensus positive GRE, and binding of GR was suggested to prevent the recruitment of positive factors that were required for gene transcription (Figure 18.2). However, transrepressive mechanisms of this nature are unlikely to explain the repression of inflammatory genes in the epithelium by glucocorticoids, as simple nGRE sites are not noted in the regulatory regions of inflammatory genes.

18.4 Inflammatory genes contain binding sites for transcriptional activators and these mediate glucocorticoid-dependent repression

The characterization of the transcriptional promoter regions of major inflammatory genes revealed key roles for transcription factors including; activator protein (AP)-1, the functionally related activating transcription factors (ATFs), CCAAT/enhancer binding protein (C/EBPβ) (NF-IL6) and, in particular, NF-κB. Importantly, glucocorticoid-dependent inhibition of inflammatory gene transcription seemed to occur via those sites (NF-κB, AP-1 etc.) that were established as being important in transcriptional activation (Barnes, 2006). Thus, glucocorticoid-dependent repression of IL-8 expression correlated with the inhibition of transcriptional activity from an IL-8 promoter construct and this effect was principally mediated via the NF-κB site in this promoter (Mukaida *et al*., 1994). Very similar stories were also developed for the rat cytokine-induced neutrophil chemoattractant (CINC/gro) gene and the human iNOS gene (Ohtsuka *et al*., 1996; Kleinert *et al*., 1996). In each of these reports, glucocorticoids repressed the DNA binding activity of NF-κB (Ohtsuka *et al*., 1996; Mukaida *et al*., 1994). Likewise, glucocorticoid-dependent repression of intercellular adhesion molecule (ICAM)-1 and E-selectin expression also involved the NF-κB site, but in these cases no effect on NF-κB DNA binding was reported (van de Stolpe *et al*., 1994; Brostjan *et al*., 1997). Such discrepancies are common and may be explained by differences in experimental protocols. For example, in A549 pulmonary cells dexamethasone showed little immediate inhibitory effect on IL-1β-induced NF-κB DNA binding induced for up to two hours, whereas stimulations of 6 h, or following long (24 h) glucocorticoid pretreatments, resulted in clear reductions in NF-κB DNA binding (Newton *et al*., 1998a). Reasons for this could involve the repression of p50/p105 (NFκB1), since mRNA expression of this gene is repressed by glucocorticoids (Newton *et al*., 1998a), and/or the induction of inhibitor of κB (IκB)α (see below).

In 1995, the expression of IκBα, the endogenous inhibitor protein for NF-κB, was shown to be increased following glucocorticoid treatment and this then led to reduced NF-κB DNA binding (Scheinman *et al*., 1995). Whilst this effect is clearly a case of glucocorticoid-dependent transactivation, since transcriptional activation of IκBα was involved (Scheinman *et al*., 1995; Wissink *et al*., 1998), the induction of IκBα is not necessary for repression of NF-κB-dependent transcription in pulmonary A549 cells (Wissink *et al*., 1998). Furthermore, glucocorticoid-dependent inducibility of IκBα in pulmonary A549 cells was considerably more modest (Wissink *et al*., 1998), indeed when stimulated with TNFα or IL-1β, there was no evidence for increased IκBα expression, and NF-κB DNA binding was unaltered over a time frame (0.5–2 h) in which glucocorticoid-dependent repression of gene expression was known to occur (Newton *et al*., 1998a; Ray *et al*., 1997). Virtually, identical results were also reported in endothelial cells suggesting that glucocorticoid-dependent transactivation of IκBα may not be a major acute anti-inflammatory mechanism in either epithelial or endothelial cells (Brostjan *et al*., 1996).

18.5 Nuclear events mediate transrepression

In order to explain the ability of glucocorticoids to transrepress NF-κB-dependent transcription, mechanisms that invoke interference, by GR, of the transcriptional activation process

have been proposed in a variety of cell types, including airway epithelial cells (see (Adcock *et al*., 2004; De Bosscher *et al*., 2003). Thus, a direct interaction between NF-κB and GR (Ray and Prefontaine, 1994; Scheinman *et al*., 1995), was suggested to account for the ability of glucocorticoids to repress NF-κB-dependent transcription (Caldenhoven *et al*., 1995), without requiring effects on either IκBα expression or on NF-κB DNA binding (De Bosscher *et al*., 1997), or site occupancy (Nissen and Yamamoto, 2000). As direct DNA binding by GR is not necessary, such forms of transrepression are described as tethering nGREs (Figure 18.2).

In parallel with, and to some extent preceding, this work on NF-κB, studies examining the glucocorticoid-dependent repression of AP-1 have led to a similar story. Thus, glucocorticoid-dependent repression of the collagenase 1 (Col 1) gene promoter was localized to an AP-1 site that was also critical in transcriptional activation (Jonat *et al*., 1990). Whilst no effect on AP-1 DNA binding was noted in one study (Jonat *et al*., 1990), inhibition of AP-1 binding was seen in a second study (Yang-Yen *et al*., 1990). Critically, this AP-1 site was sufficient to confer steroid-dependent repression, and a direct interaction between AP-1 and GR was proposed to account for the mutual antagonism observed between these two pathways (Jonat *et al*., 1990; Yang-Yen *et al*., 1990). The effect of glucocorticoids on AP-1 DNA binding was subsequently readdressed and the occupancy of AP-1 sites was found not to be altered suggesting, as with NF-κB, a direct interference with transcription as the mechanism of inhibition (Konig *et al*., 1992).

One apparent consequence of transrepression by direct interference was the observation that both AP-1 and NF-κB showed a mutual antagonism with GR and this was suggested to result from competition for co-activators, in particular, CREB binding protein (CBP) (Sheppard *et al*., 1998; McKay and Cidlowski, 2000). However, other studies dispel this notion and instead suggest that interference with the basal transcriptional apparatus is the explanation for transrepression of both NF-κB and AP-1 (De Bosscher *et al*., 2000, 2001), possibly via recruitment of the p160 family member, GR interacting protein (GRIP)

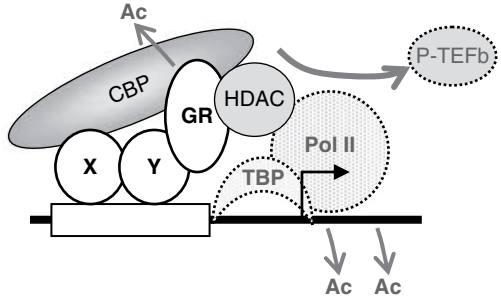


Figure 18.3 Transcriptional repression by GR in a tethering conformation. At tethering nGREs, as may occur with NF-κB, or AP-1, repression is achieved via the recruitment of histone deacetylase and the loss of a regulatory kinase. GR exerts a repressive effect by recruiting p160 family members (not shown) and one or more histone deacetylases (HDAC), which deacetylate the DNA in the promoter region leading to closing of the chromatin structure and GR to promote interaction with NF-κB. Acetyl groups (Ac) are shown leaving the promoter and GR. Finally, GR-dependent loss of the Pol II C-terminal domain kinase, P-TEFb, reduces phosphorylation of Pol II and reduces transcription of specific target genes

(Rogatsky *et al*., 2001, 2002). Thus, phosphorylation of the C-terminal domain of RNA polymerase (Pol) II was prevented by dexamethasone, possibly following GR-dependent loss of a regulatory kinase complex, and this mediated promoter-selective inhibition of NF-κB-dependent genes (Nissen and Yamamoto, 2000; Luecke and Yamamoto, 2005). In keeping with the idea of post-translational modification, and the fact that histone acetylation is necessary for activated transcription of inflammatory genes, glucocorticoids were shown to decrease the acetylation induced by IL-1β at the human granulocyte macrophage-colony stimulating factor (GM-CSF) promoter (Ito *et al*., 2000). This process should lead to a less favourable promoter conformation and appears to operate by reducing CBP-associated histone acetylase (HAT) activity, recruitment of histone deacetylase (HDAC) 2 to the p65-CBP complex, and possibly by promoting deacetylation of GR itself (Ito *et al*., 2000, 2006) (Figure 18.3).

18.6 Transactivation plays an important anti-inflammatory role

In the above sections, evidence is presented for the existence of mechanisms of transrepression that, for example, lead to reduced activity of NF-κB and AP-1 and then to reduced expression of inflammatory genes. However, to say that this type of repression explains all the anti-inflammatory effects of glucocorticoids is to overlook large bodies of data that implicate critical roles for both post-transcriptional control processes and glucocorticoid-dependent gene expression (Newton, 2000; Stellato, 2004; Abraham and Clark, 2006). Furthermore, in our assessment, current data do not convincingly exclude GR-dependent gene activation from playing important roles in the anti-inflammatory effects of glucocorticoids. Given this point of view, the question as to the relative importance of transcriptional repression (transrepression) by glucocorticoids, as opposed to other forms of repression, becomes relevant. In this respect, it is critical to recall that both the potency and efficacy of GR-dependent transcription can be modulated by GR numbers relative to co-activator/co-repressor numbers (Szapary *et al*., 1999; Wang *et al*., 2004). Therefore, studies in which GR is overexpressed, as commonly occurs in the analysis of transrepression, are likely to produce an overrepresentation of that glucocorticoid-dependent function. Consequently, it is important to consider the endogenous, or physiological, levels of GR in assessing the relative contribution of particular mechanisms to the overall repression.

Despite the above focus on transrepression, in A549 pulmonary epithelial cells dexamethasone-dependent transrepression of an NF-κB reporter was no more that 40 or 50 per cent and this correlated with the actual transcription rate of known NF-κB-dependent genes, including COX-2 and IL-8, as assessed by both nuclear run on transcription and analysis of the transient accumulation of unspliced nuclear RNA intermediates (Newton *et al*., 1998a, 1998b; Chivers *et al*., 2006). Similarly, analysis of IL-8 transcription rate in primary human airway epithelial cells was also not substantially repressed by dexamethasone, yet IL-8 expression was significantly reduced (Chang *et al*., 2001). Again, in A549 cells the induction of IL-11 expression by TGFβ was strongly repressed by dexamethasone via mechanisms that did not primarily involve reductions in IL-11 transcription rate (Wang *et al*., 1999). Thus, considerable data exists for a key role of post-transcriptional mechanisms of repression by glucocorticoids in a variety of cell types that includes the pulmonary epithelium.

18.7 Post-transcriptional effects and a role for glucocorticoid-dependent gene expression

The suggestion that transcriptional repression by glucocorticoids does not account for the full repressive effect of these compounds observed at the level of gene expression supports the prospect of additional repressive mechanisms. Thus, post-transcriptional processes, in particular mRNA de-stabilization, have been proposed in the glucocorticoid-dependent repression of IL-1 and LPS-induced COX-2 expression in multiple cell systems (Newton *et al*., 1998b; Ristimaki *et al*., 1996; Lasa *et al*., 2001), mitogen-induced IL-4Rα expression (Mozo *et al*., 1998), TNFα-induced expression of GM-CSF, IL-8 and IL-6 in fibroblasts (Tobler *et al*., 1992), IL-1β expression in LPS and phorbol ester-stimulated monocytes (Kern *et al*., 1988; Lee *et al*., 1988), TGFβ-induced IL-11 (Wang *et al*., 1999) and LPS-induced iNOS expression in macrophage (Korhonen *et al*., 2002). In terms of inflammatory gene expression, these results are, perhaps, not surprising as mRNA stabilization is a major regulator of gene expression and it is logical that these processes would also be targeted by glucocorticoids in epithelial cells (Stellato, 2004; Newton, 2000; Mata *et al*., 2005; Fan *et al*., 2005). Importantly, numerous investigators show that the ability of glucocorticoids to repress the expression of target genes, when added after an inducing stimulus such as IL-1β or LPS, is prevented by co-incubation with inhibitors of transcription or translation (for examples see Lee *et al*. (1988), Ristimaki *et al*. (1996), Korhonen *et al*. (2002), Tobler *et al*. (1992), Chang *et al*. (2001), Newton *et al*. (1998b) and Staples *et al*. (2003)). Thus a key role for *de novo* glucocorticoid-dependent gene expression is implicated in the glucocorticoid-dependent repression of these inflammatory genes (Newton, 2000). In this regard, AUUUA motifs, or AU-rich elements (ARE), which are located in the 3′ untranslated regions (UTR) of many unstable RNAs, have proved to be critical in signal-induced mRNA stabilization of inflammatory genes (Fan *et al*., 2005). Many unstable cytokine and inflammatory gene mRNAs, including, for example, IL-6, IL-8 and COX-2, are stabilized via their AREs due to the actions of the p38 mitogen activated protein kinase (MAPK) pathway (Winzen *et al*., 1999; Lasa *et al*., 2000, and see Clark *et al*. (2003) and Dean *et al*. (2004)). Furthermore, in addition to mediating mRNA stabilization, AREs can also mediate glucocorticoid-dependent mRNA decay (Peppel *et al*., 1991; Lasa *et al*., 2001). While numerous ARE-binding proteins have been identified, the details of the mRNA stabilization and destabilization processes remain unclear (Dean *et al*., 2004). However, the ARE binding protein, tristetraprolin (TTP) is believed to regulate gene expression, for example TNFα (Mahtani *et al*., 2001), by promoting deadenylation and mRNA destabilization (Lai *et al*., 1999). Mice lacking TTP show elevated levels of TNFα and develop chronic inflammation in a manner that is consistent with a role for TTP the normal feedback control of inflammatory gene expression (Brook *et al*., 2006; Carballo *et al*., 1998). In the context of glucocorticoid actions, dexamethasone has been reported to induce TTP expression in pulmonary A549 cells, raising the possibility that the normal regulatory roles of TTP are exploited by anti-inflammatory pathways (Smoak and Cidlowski, 2006).

18.8 MKP-1, an anti-inflammatory glucocorticoid-inducible gene

The involvement of the p38 MAPK in mRNA stabilization of inflammatory genes provides a mechanistic explanation for mRNA destabilization following glucocorticoid treatment

(Clark, 2003). Glucocorticoids block the activation of p38 MAPK via a process that is prevented by transcriptional inhibitors (Lasa *et al*., 2001). This suggests the involvement of glucocorticoid-dependent gene expression and, indeed, glucocorticoids very profoundly induce the expression of MAPK phosphatase (MKP)-1, also known as dual specificity phosphatase 1 (Lasa *et al*., 2002; Chen *et al*., 2002; Kassel *et al*., 2001). Dephosphorylation of p38 MAPK by MKP-1 will destabilize mRNA and lead to repression of cytokine expression (Lasa *et al*., 2002; Chen *et al*., 2002).

The effects of glucocorticoid-dependent induction of MKP-1 are by no means limited, however, to destabilization of p38 MAPK-dependent mRNAs. The p38 pathway is implicated in the activation of various transcription factors, including ATF-1, ATF-2, as well as directly in the expression AP-1 components (see (Newton and Holden, 2003). Therefore, the inhibition of p38, which inhibits AP-1 transcriptional activity (Wesselborg *et al*., 1997), by the glucocorticoid-dependent induction of MKP-1 may lead to the transcriptional repression of many inflammatory genes, for example E-selectin, that are regulated by AP-1 and/or ATF factors (Furst *et al*., 2007). Likewise, many reports indicate positive effects of the p38 MAPK on NF-κB-dependent transactivation (Wesselborg *et al*., 1997, Newton and Holden, 2003). Thus, increased expression of MKP-1 could negatively impact on the expression of NF-κB-dependent genes. Furthermore, since analysis of selective p38 MAPK inhibitors specifically implicated a role for this kinase in the translational control of inflammatory cytokines (Lee *et al*., 1994), it is highly likely that glucocorticoids acting via MPK-1 will also inhibit cytokine translation in the pulmonary epithelium.

MKP-1 may also act on other members of the MAPK family of kinases. For example the extracellular regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs) have also be shown to be MKP-1 substrates and may, therefore, be subject to glucocorticoid-dependent inhibition by this phosphatase (Franklin and Kraft, 1997; Kassel *et al*., 2001; Slack *et al*., 2001). In addition to preventing serum response element (SRE) and Elk-1 activation (Wu *et al*., 2005), MPK-1-dependent inhibition of JNK may directly repress AP-1 dependent transcription (Liu *et al*., 1995). Furthermore, the JNK pathway is implicated in the translational control of TNFα biosynthesis induced by LPS, and this can be targeted by glucocorticoids (Swantek *et al*., 1997).

18.9 Other glucocorticoid-inducible genes with anti-inflammatory potential in the epithelium

Whilst MKP-1 has received considerable attention as an anti-inflammatory glucocorticoid-inducible gene (Abraham and Clark, 2006), the fact that glucocorticoid-dependent repression of inflammatory gene expression is only partially lost in MKP-$1^{-/-}$ animals indicates the existence of further repressive mechanisms (Abraham *et al*., 2006). In this context there are now numerous other glucocorticoid-inducible genes that have been described that may also be expected to show anti-inflammatory effect. For example, glucocorticoids induce the expression of the Clara cell secretory 10 kDa protein (CC10), in part via the activation of the CCAAT/enhancer binding proteins (C/EBPs) (Berg *et al*., 2005; Cassel *et al*., 2000). Furthermore, this gene is protective in the context of pulmonary allergic inflammation (Chen *et al*., 2001). Likewise glucocorticoid-inducible leucine zipper (GILZ) is a transcriptional regulator that is very highly glucocorticoid-inducible in airway epithelial cells (Eddleston *et al*., 2007). Importantly, GILZ is implicated in the repression of AP-1 and NF-κB, so

an anti-inflammatory activity for this protein by repressing classical inflammatory genes is fairly clear (Mittelstadt and Ashwell, 2001; Eddleston *et al*., 2007).

18.10 Effect of glucocorticoids on lipid mediator production by epithelial cells

The airway epithelium is a well established source of lipid mediators, which include cyclooxygenase and 15-lipoxygenase products as well as platelet-activating factor (PAF) (Martin *et al*., 1997). More recently the airway epithelium has been found to possess the 5-lipoxygenase pathway and may synthesize leukotrienes (LT)C_4 and B_4 (Jame *et al*., 2007). Furthermore the increased expression of the 5-lipoxygenase enzyme, following stimulation with Ca^{2+} ionophore, appeared to be down-regulated by dexamethasone. More traditionally the production of cyclooxygenase products, for example prostaglandin (PG)E_2 and $PGF_{2\alpha}$ is induced by inflammatory stimuli that may include pro-inflammatory cytokines (IL-1β, TNFα) and elevation of intracellular Ca^{2+} levels, for example following stimulation with bradykinin (Saunders *et al*., 1999; Newton *et al*., 2002). In this context the expression of COX-2 is necessary for induced prostaglandin production, and this enzyme is very profoundly repressed by glucocorticoids via both the down-regulation of COX-2 gene transcription and mRNA destabilization (Mitchell *et al*., 1994; Newton *et al*., 1998b). In addition, the downstream enzyme, microsomal prostaglandin E synthase (mPGES), which is required for PGE_2 production, is both induced by pro-inflammatory cytokines and repressed by dexamethasone (Thoren and Jakobsson, 2000). However, glucocorticoids also exert a profound repressive effect upstream on the release of arachidonic acid from epithelial cells, and this will contribute to the reduction of prostaglandin production (Chivers *et al*., 2004). Mechanisms for this effect are varied and traditionally explained by the ability of glucocorticoids to induce lipocortin 1 expression and, thereby, inhibit cytosolic phospholipase (cPL) A_2 activity (Flower and Rothwell, 1994). However, other more rapid mechanisms may also exist which do not involve genomic processes (Croxtall *et al*., 2000, 2002). Finally, the activation of $cPLA_2$ and the release of arachidonic acid requires the activation of both the ERK and p38 MAPKs (Newton *et al*., 2000). As noted above, the ability of glucocorticoids to induce the expression of MKP-1 to dephosphorylate and switch off MAPKs provides an additional mechanism of repression of prostaglandin production. Furthermore these effects, on both the regulation and activation of $cPLA_2$, may explain the reductions in PAF or other downstream metabolites that are generated via other pathways (15-lipoxygenase, 5-lipoxygenase) which also utilize this enzyme (Schleimer, 1993).

18.11 Remodelling, viruses, glucocorticoids and the epithelium

In the above sections, we have seen how glucocorticoids are highly effective in combating inflammatory responses of the epithelium. In the past, the ability to prevent inflammation would be expected to lead to attenuated airway remodelling that is commonly associated with diseases such as asthma. However, more recently, airway remodelling has been described prior to the onset of symptoms, as well as in young children, suggesting that remodelling

may not necessarily follow inflammation, but could develop simultaneously with, or even precede, inflammation (Tang *et al*., 2006). Therefore, preventing inflammation may not necessarily lead to improvements in remodelling and it is relevant to assess the effect of glucocorticoids directly on remodelling responses. In this context, the epithelium is known to produce remodelling factors such as TGFβ and VEGF, and the expression of these mediators is reduced by glucocorticoids (Beckett and Howarth, 2003). Furthermore, basement membrane thickening is also reduced by glucocorticoid treatment (Hoshino *et al*., 1998). Likewise goblet cell hyperplasia and mucus hypersecretion are prominent features of airway remodelling and again these responses are reversed by glucocorticoid treatment, primarily due to effects on the underlying inflammation, but also due to a possible direct repression of mucin gene expression (Rogers, 2004; Lu *et al*., 2005; Chen *et al*., 2006).

With the idea that remodelling may occur at the same time, or even before, symptoms in asthma, the concept arises that certain respiratory viruses may promote remodelling or other phenotypic changes that could lead to later airway disease (Holtzman *et al*., 2002; Proud and Chow, 2006). In this context, numerous reports suggest that glucocorticoids are of lesser effect in the context of viral exacerbations of airway disease, and overall only a modest benefit is suggested (Proud and Chow, 2006). Thus, while some studies report little effect of dexamethasone on inflammatory mediator production, or ICAM-1 expression, by epithelial cells infected with either respiratory syncytial virus or rhinovirus (Grunberg *et al*., 2000; Carpenter *et al*., 2002), contradictory reports exist (Suzuki *et al*., 2000). Finally, expression of VEGF from epithelial cells is enhanced by rhinovirus and this is blocked by the glucocorticoid, fluticasone (Volonaki *et al*., 2006). Thus remodelling changes induced by viral infection, which may later predispose to later disease, could be attenuated by concurrent glucocorticoid therapy.

18.12 Ion and other channels in the epithelium and the effect of glucocorticoids

Like the collecting ducts in the kidney, the airway epithelium is capable of vectorial transport of both solutes and water between the airway lumen and the sub-epithelial layers (Matthay *et al*., 2002; Folkesson and Matthay, 2006) (Figure 18.4). The basic paradigm is that active transport of both Na^+ and Cl^- from the lumen, through the epithelial/alveolar cells to the basolateral side, promotes the passive movement of water into the extracellular space (see Chapter 5). The pumping of Na^+ from the airway lumen predominantly occurs via the epithelial sodium channel (ENaC), which exists as a number of distinct (α, β, γ) subunits, as well as via other channels such as cyclic nucleotide gated channels (CNG) in the apical membrane (Figure 18.4). In order to prevent Na^+ build-up within the cell and to maintain a concentration gradient, Na^+/K^+ ATPases are positioned in the basolateral membrane. These pump Na^+ out of the cell into the interstitial space and bring K^+ into the cell. This results in a net movement of Na^+ across the cell. Flow of water across the cell may be facilitated by the expression of water channels known as aquaporins (AQPs) (Matthay *et al*., 2002). Thus the expression of AQP3 is found in epithelial cells of the large airway, whereas AQP4 appears to be general to all epithelial cells in the airway and AQP5 is found on the apical membranes of type I alveolar cells, as well as more generally in airway and alveolar cells (Borok and Verkman, 2002). By contrast, AQP1 is predominantly found on endothelial cells, but there are suggestions of expression on alveolar

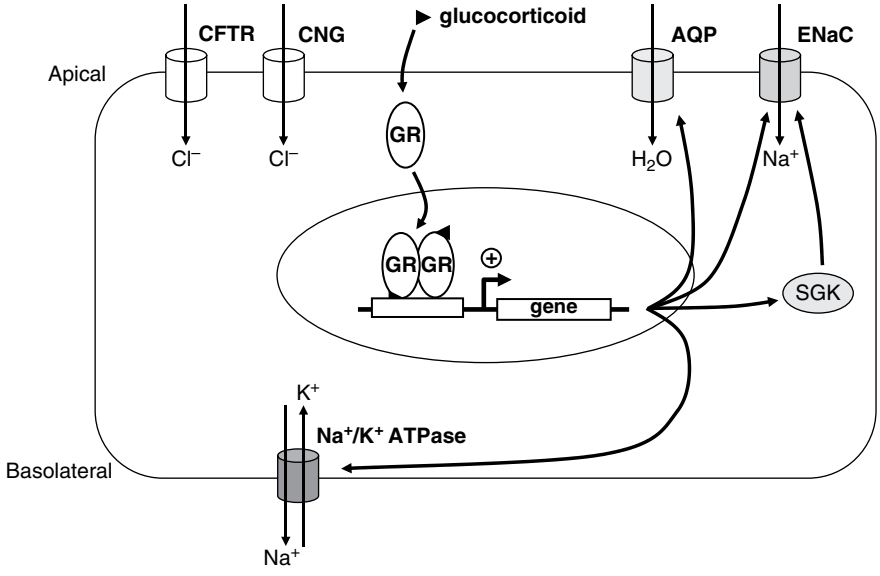


Figure 18.4 Regulation of airway fluid clearance by glucocorticoids. Airway and alveolar cells sit with their apical surface towards the lumen and the basolateral surface on a basement membrane that links them to the interstitial part of the lung. The apical surface expresses the epithelial sodium pump (ENaC), which is responsible for bringing Na^+ into the cell. Parallel uptake of Cl^- via various chloride channels, which may include the cystic fibrosis trans-membrane receptor (CFTR), cyclic nucleotide-gated channels (CNG), or other channels, maintains electrical neutrality. Sodium is removed from the cell by the Na^+/K^+ ATPase, which pumps Na^+ into the interstitial space. The transfer of water may be facilitated by the presence of water channels, or aquaporins (AQP). This process is markedly upregulated by glucocorticoids, which activate gene expression of components of ENaC, the Na^+/K^+ ATPase and certain AQPs. Furthermore, glucocorticoids upregulate the serum and glucocorticoid-inducible kinase (SGK), which can phosphorylate a number of targets including ENaC to stimulate ENaC activity and reduce degradation. The upregulation of ENaC, AQP, Na^+/K^+ ATPase or SGK is depicted via a simple GRE, but this may not necessarily be the case. Note: this figure does not attempt to represent the full balance of the electrochemical, osmotic and other gradients that may exist as only selected pumps, channels or ions/molecules are depicted

cells (Borok and Verkman, 2002). In the kidney, many of these processes are regulated by the mineralocorticoid receptor following the binding of aldosterone. However, in the lung, glucocorticoids, for example from inhaled medications, may bind to GR and elicit similar effects, in part due to the highly homologous nature of these two receptors in the DNA-binding domains and the commonality of their DNA recognition sites (Lu *et al*., 2006). In A549 type II cells, dexamethasone increased the expression of AQP3 (Tanaka *et al*., 1997), whereas AQP1 expression is more generally upregulated by glucocorticoid in rat lungs (King *et al*., 1996). Together, these results may help to explain the increased alveolar fluid clearance that is observed following dexamethasone treatment (Folkesson *et al*., 2000). However, glucocorticoids also show an ability to positively modulate the sodium pump and Na/K ATPase in the epithelial cell (Matthay *et al*., 2002). Indeed, components of both ENaC and the Na/K ATPase are upregulated by glucocorticoids in alveolar and epithelial

cells (Dagenais *et al*., 2001; Otulakowski *et al*., 2006; Chalaka *et al*., 1999). Interestingly, viral stimulation leads to reduced expression of AQP1 and 5, and TNFα reduces ENaC expression. Such effects could have considerable relevance to pathological edema in infection or inflammation (Towne *et al*., 2000; Dagenais *et al*., 2006). However, the upregulation of AQPs by glucocorticoids (above), and the finding that TNFα-reduced ENaC expression was reversed by glucocorticoids indicates the potential for a positive effect of glucocorticoids on lung edema (Dagenais *et al*., 2006). The regulation of Na^+, and thereby, water influx can be further modified by the serum and glucocorticoid-inducible kinase (SGK) 1, which, in addition to stimulating ENaC expression, is capable of phosphorylating ENaC to enhance activity (Pearce and Kleyman, 2007). However, this kinase is also induced by glucocorticoids in the lung and this provides a further level of positive control by glucocorticoids on fluid clearance from the airway/alveolar space (Itani *et al*., 2002). Thus, glucocorticoids offer a variety of mechanisms and pathways by which increased fluid clearance from the airway/alveolar space may be achieved.

18.13 Maturation of the fetal airway epithelium

Infant respiratory distress syndrome (RDS) is associated with preterm birth and is, in part, due to the lack of maturity of the airway epithelium (Bolt *et al*., 2001). Key components of this syndrome are the lack of surfactant proteins and the inability to adequately clear fluid from the airway. Clues as to the mechanism for this effect are provided by GR-deficient mice, which show reduced expression of the surfactant proteins (SPs), SP-A and C, as well as the water channels AQP1, AQP5 and T1α (Cole *et al*., 2004). This is consistent with the long-standing clinical practice of glucocorticoid administration to rapidly promote fetal lung maturation (Bolt *et al*., 2001). The ability of glucocorticoids to promote fluid clearance by the epithelial and alveolar epithelium is well documented and this appears to hold true for the fetal lung epithelium, since glucocorticoids both improve RDS and increase the expression of ENaCα (Helve *et al*., 2004; Otulakowski *et al*., 2006). Furthermore, glucocorticoids, in the context of a cAMP elevating stimulus, may also increase the expression of the surfactant proteins SP-A, B, C and D (Gonzales *et al*., 2002; Wade *et al*., 2006). Since deficiencies in surfactant protein expression are known to cause RDS, such effects of glucocorticoids are likely to be highly beneficial on the epithelium in the context of pre-term delivery, where reductions in airway inflammation may also be advantageous (Bolt *et al*., 2001).

18.14 Non-genomic actions of glucocorticoids

The possibility that steroid hormones may act via non-genomic mechanisms is not a new concept as certain responses, which can occur within minutes of steroid administration, have been known for many years (Losel *et al*, 2003; Falkenstein *et al*., 2000). Losel and Wehling list a number of criteria that may be helpful in defining or identifying non-genomic actions for steroid hormones (Table 18.1). For example, in a guinea pig model of allergic asthma, the synthetic glucocorticoid, budesonide, inhibited certain allergic reactions within 10 minutes of administration, a time frame inconsistent with current thinking regarding genomic mechanisms of action (Zhou *et al*., 2003). Furthermore, the finding that glucocorticoids attenuate itch responses in the context of allergic rhinitis (in humans) provides strong support for the

Table 18.1 Characteristics of a response which may suggest a non-genomic mode of glucocorticoid action

- Occurs in cells which do not posses a functional nucleus. e.g. platelets, erythrocytes.
- The response is refractory to inhibition of transcription (e.g. actinomycin D) or translation (e.g. cycloheximide).
- Occurs very rapidly, i.e. within minutes, which is generally thought to be inconsistent with altered gene expression via changes in transcription and/or translation.
- May be elicited by agonists that cannot access the interior of the cell, i.e. is then inconsistent with binding to a receptor that then translocates to the nucleus.

Criteria derived from Losel and Wehling (2003).

previous study (Tillmann *et al*., 2004). Certainly, the rapid onset of action is highly suggestive of a non-genomic action, although it is worth considering that some genes (e.g. c-fos) are capable of induction within minutes of a stimulus. At the cellular level in human bronchial epithelial cells, it is interesting to note that dexamethasone has been suggested to stimulate the Na^+/H^+ exchanger and inhibit ATP-induced Ca^{2+}-dependent Cl^- secretion via rapid gene expression-independent processes (Urbach *et al*., 2002, 2006; Verriere *et al*., 2005). A further effect that may have biological and pharmacological significance is that glucocorticoids can prevent the stimulation of both PLA_2 activity and arachidonic acid release from A549 cells via a process that is refractory to transcriptional inhibition (Croxtall *et al*., 2000). Importantly, the rank orders of potencies for a number of clinically relevant glucocorticoids was totally different in respect of this, and other outputs, when compared against classical anti-inflammatory effects such as the repression of COX-2 expression (Croxtall *et al*., 2002). Such data is consistent with suggestions of pertussis toxin-sensitive modes of glucocorticoid action and raises the possibility of membrane associated, non-GR, receptors for glucocorticoids that could even include G-protein coupled receptors (Qiu *et al*., 2003; Losel *et al*., 2003; Stellato, 2004; Tasker *et al*., 2006).

18.15 Summary

In conclusion, glucocorticoids acting on the airway epithelium can modulate a variety of responses by eliciting changes in gene expression, as well as via direct effects. In terms of the anti-inflammatory effects of glucocorticoids, there is considerable data concerning the ability to transrepress key transcription factors such as NF-κB or AP-1. There also is an increasing body of data that indicates the importance of glucocorticoid-inducible genes, which may then act to transcriptionally, post-transcriptionally, translationally or post-transcriptionally to dampen down the expression or activity of inflammatory genes (Figure 18.5). These processes are now well established in a number of cell types, but do require considerable validation in the context of the airway epithelium. In respect to the control of fluid and salt balance, as well as lung maturation, glucocorticoids induce the expression of a number of key genes that are critical to these processes. Finally, there is increasing evidence for non-genomic effects of glucocorticoids, and these may even involve receptor-dependent

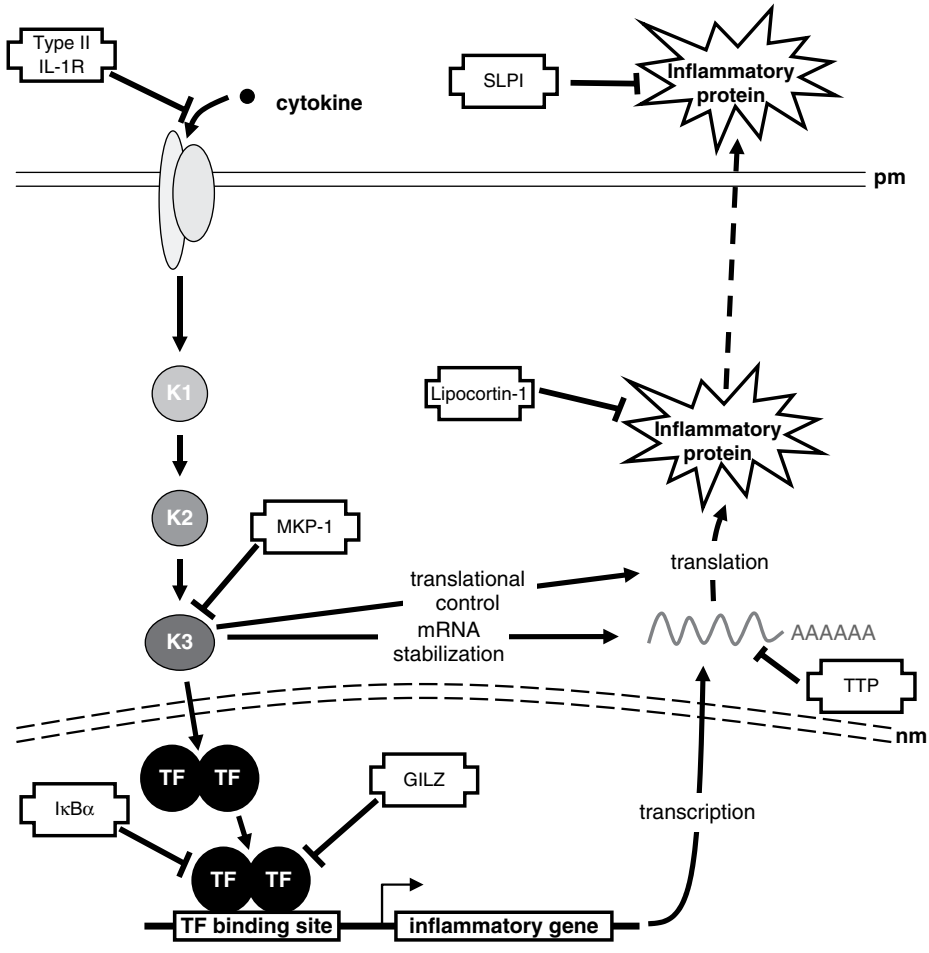


Figure 18.5 Impact of glucocorticoid-inducible genes on inflammatory gene expression. A schematic representation of the signalling cascades leading to inflammatory gene expression is depicted with possible targets and sites of action for putative anti-inflammatory glucocorticoid-inducible genes. Activation of a pro-inflammatory cascade, following binding of cytokine to its cognate receptor in the plasma membrane (pm), is shown occurring via a number of kinases (K1–3). The signal crossed the nuclear membrane (nM) and leads to transcription factor (TF) activation and the production of inflammatory gene mRNA. Under the influence of further kinase cascades (here K1–3), the mRNA is stabilized and translated into protein. Finally, many proteins are exported into the extracellular space for function. Sites of action of glucocorticoid-inducible genes are indicated. The glucocorticoid-inducible type II IL-1 receptor (IL-1R) acts as a decoy receptor to prevent activation of the cell by IL-1. Mitogen-activated protein kinase phosphatase (MKP)-1 is an inhibitor of the MAP kinase family and therefore impacts on numerous cellular mechanisms including activation of transcription, mRNA stability and translation. Inhibitor of κB (IκB)α and GILZ inhibit key inflammatory transcription factors (NF-κB and AP-1). Tristetraprolin (TTP) promotes deadenylation and degradation of mRNA. Lipocortin-1 inhibits PLA_2. Finally secretory leukocyte protease inhibitor (SLPI) is a potent inhibitor of serine proteases

events other than by GR. In general, neither non-genomic effects, nor anti-inflammatory effects occurring via gene induction, are currently considered in sufficient detail in terms of potential therapies for the lung. These areas require considerable investigation and could offer an opportunity for improving treatment modalities that act on the airway epithelium.

Acknowledgements

Work in Dr Newton's laboratory is supported by the Canadian Institutes of Health Research (CIHR) and the Alberta Heritage Foundation for Medical Research (AHFMR).

References

Abbinante-Nissen JM, Simpson LG, and Leikauf GD (1995) Corticosteroids increase secretory leukocyte protease inhibitor transcript levels in airway epithelial cells. *Am J Physiol* **268**: L601–L606.

Abraham SM and Clark AR (2006) Dual-specificity phosphatase 1: a critical regulator of innate immune responses. *Biochem Soc Trans* **34**: 1018–1023.

Abraham SM, Lawrence T, Kleiman A, Warden P, Medghalchi M, Tuckermann J, Saklatvala J, and Clark AR (2006) Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *J Exp Med* **203**: 1883–1889.

Adcock IM, Ito K, and Barnes PJ (2004) Glucocorticoids: effects on gene transcription. *Proc Am Thorac Soc* **1**: 247–254.

Adkins KK, Levan TD, Miesfeld RL, and Bloom JW (1998) Glucocorticoid regulation of GM-CSF: evidence for transcriptional mechanisms in airway epithelial cells. *Am J Physiol* **275**: L372–L378.

Atsuta J, Plitt J, Bochner BS, and Schleimer RP (1999) Inhibition of VCAM-1 expression in human bronchial epithelial cells by glucocorticoids. *Am J Respir Cell Mol Biol* **20**: 643–650.

Barnes PJ (2006) How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *Br J Pharmacol* **148**: 245–254.

Beckett PA and Howarth PH (2003) Pharmacotherapy and airway remodelling in asthma? *Thorax* **58**: 163–174.

Berg T, Didon L, Barton J, Andersson O, and Nord M (2005) Glucocorticoids increase C/EBPbeta activity in the lung epithelium via phosphorylation. *Biochem Biophys Res Commun* **334**: 638–645.

Bolt RJ, van Weissenbruch MM, Lafeber HN, and Delemarre-van de Waal HA (2001) Glucocorticoids and lung development in the fetus and preterm infant. *Pediatr Pulmonol* **32**: 76–91.

Borok Z and Verkman AS (2002) Lung edema clearance: 20 years of progress: invited review: Role of aquaporin water channels in fluid transport in lung and airways. *J Appl Physiol* **93**: 2199–2206.

Brook M, Tchen CR, Santalucia T, McIlrath J, Arthur JS, Saklatvala J, and Clark AR (2006) Posttranslational regulation of tristetraprolin subcellular localization and protein stability by P38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. *Mol Cell Biol* **26**: 2408–2418.

Brostjan C, Anrather J, Csizmadia V, Stroka D, Soares M, Bach FH, and Winkler H (1996) Glucocorticoid-mediated repression of NFkappaB activity in endothelial cells does not involve induction of IkappaBalpha synthesis. *J Biol Chem* **271**: 19612–19616.

Brostjan C, Anrather J, Csizmadia V, Natarajan G, and Winkler H (1997) Glucocorticoids inhibit E-selectin expression by targeting NF-kappaB and not ATF/c-Jun. *J Immunol* **158**: 3836–3844.

Caldenhoven E, Liden J, Wissink S, van de SA, Raaijmakers J, Koenderman L, Okret S, Gustafsson JA, and van der Saag PT (1995) Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol Endocrinol* **9**: 401–412.

Carballo E, Lai WS, and Blackshear PJ (1998) Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science* **281**: 1001–1005.

Carpenter LR, Moy JN, and Roebuck KA (2002) Respiratory syncytial virus and TNF alpha induction of chemokine gene expression involves differential activation of Rel A and NF-kappa B1. *BMC Infect Dis* **2**: 5.

Cassel TN, Nordlund-Moller L, Andersson O, Gustafsson JA, and Nord M (2000) C/EBPalpha and C/EBPdelta activate the Clara cell secretory protein gene through interaction with two adjacent C/EBP-binding sites. *Am J Respir Cell Mol Biol* **22**: 469–480.

Chalaka S, Ingbar DH, Sharma R, Zhau Z, and Wendt CH (1999) Na(+)-K(+)-ATPase gene regulation by glucocorticoids in a fetal lung epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* **277**: L197–L203.

Chang MM, Juarez M, Hyde DM, and Wu R (2001) Mechanism of dexamethasone-mediated interleukin-8 gene suppression in cultured airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **280**: L107–L115.

Chen LC, Zhang Z, Myers AC, and Huang SK (2001) Cutting edge: altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-KDa protein. *J Immunol* **167**: 3025–3028.

Chen P, Li J, Barnes J, Kokkonen GC, Lee JC, and Liu Y (2002) Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *J Immunol* **169**: 6408–6416.

Chen Y, Nickola TJ, DiFronzo NL, Colberg-Poley AM, and Rose MC (2006) Dexamethasone-mediated repression of MUC5AC gene expression in human lung epithelial cells. *Am J Respir Cell Mol Biol* **34**: 338–347.

Chivers JE, Cambridge LM, Catley MC, Mak JC, Donnelly LE, Barnes PJ, and Newton R (2004) Differential effects of RU486 reveal distinct mechanisms for glucocorticoid repression of prostaglandin E release. *Eur J Biochem* **271**: 4042–4052.

Chivers JE, Gong W, King EM, Seybold J, Mak JC, Donnelly LE, Holden NS, and Newton R (2006) Analysis of the dissociated steroid, RU24858, does not exclude a role for inducible genes in the anti-inflammatory actions of glucocorticoids. *Mol Pharmacol* **70**: 2084–2095.

Clark AR (2003) MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? *J Endocrinol* **178**: 5–12.

Clark AR, Dean JL, and Saklatvala J (2003) Post-transcriptional regulation of gene expression by mitogen-activated protein kinase P38. *FEBS Lett* **546**: 37–44.

Cole TJ, Solomon NM, Van Driel R, Monk JA, Bird D, Richardson SJ, Dilley RJ, and Hooper SB (2004) Altered epithelial cell proportions in the fetal lung of glucocorticoid receptor null mice. *Am J Respir Cell Mol Biol* **30**: 613–619.

Croxtall JD, Choudhury Q, and Flower RJ (2000) Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. *Br J Pharmacol* **130**: 289–298.

Croxtall JD, Van Hal PT, Choudhury Q, Gilroy DW, and Flower RJ (2002) Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. *Br J Pharmacol* **135**: 511–519.

Dagenais A, Denis C, Vives MF, Girouard S, Masse C, Nguyen T, Yamagata T, Grygorczyk C, Kothary R, and Berthiaume Y (2001) Modulation of alpha-ENaC and alpha1-Na + -K + -ATPase by CAMP and dexamethasone in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **281**: L217–L230.

Dagenais A, Frechette R, Clermont ME, Masse C, Prive A, Brochiero E, and Berthiaume Y (2006) Dexamethasone inhibits the action of TNF on ENaC expression and activity. *Am J Physiol Lung Cell Mol Physiol* **291**: L1220–L1231.

Davies TH, Ning YM, and Sanchez ER (2002) A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. *J Biol Chem* **277**: 4597–4600.

De Bosscher K, Schmitz ML, Vanden Berghe W, Plaisance S, Fiers W, and Haegeman G (1997) Glucocorticoid-mediated repression of nuclear factor-kappaB-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci U S A* **94**: 13504–13509.

De Bosscher K, Vanden Berghe W, Vermeulen L, Plaisance S, Boone E, and Haegeman G (2000) Glucocorticoids repress NF-kappaB-driven genes by disturbing the interaction of P65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc Natl Acad Sci U S A* **97**: 3919–3924.

De Bosscher K, Vanden Berghe W, and Haegeman G (2001) Glucocorticoid repression of AP-1 is not mediated by competition for nuclear coactivators. *Mol Endocrinol* **15**: 219–227.

De Bosscher K, Vanden Berghe W, and Haegeman G (2003) The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev* **24**: 488–522.

Dean JL, Sully G, Clark AR, and Saklatvala J (2004) The involvement of AU-rich element-binding proteins in P38 mitogen-activated protein kinase pathway-mediated MRNA stabilisation. *Cell Signal* **16**: 1113–1121.

Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC, and Zuraw BL (2007) The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *J Allergy Clin Immunol* **119**: 115–122.

Falkenstein E, Tillmann HC, Christ M, Feuring M, and Wehling M (2000) Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. *Pharmacol Rev* **52**: 513–556.

Fan J, Heller NM, Gorospe M, Atasoy U, and Stellato C (2005) The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. *Eur Respir J* **26**: 933–947.

Flower RJ and Rothwell NJ (1994) Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol Sci* **15**: 71–76.

Folkesson HG and Matthay MA (2006) Alveolar epithelial ion and fluid transport: recent progress. *Am J Respir Cell Mol Biol* **35**: 10–19.

Folkesson HG, Norlin A, Wang Y, Abedinpour P, and Matthay MA (2000) Dexamethasone and thyroid hormone pretreatment upregulate alveolar epithelial fluid clearance in adult rats. *J Appl Physiol* **88**: 416–424.

Fragaki K, Kileztky C, Trentesaux C, Zahm JM, Bajolet O, Johnson M, and Puchelle E (2006) Down-regulation by a long-acting beta2-adrenergic receptor agonist and corticosteroid of *Staphylococcus aureus*-induced airway epithelial inflammatory mediator production. *Am J Physiol Lung Cell Mol Physiol* **291**: L11–L18.

Franklin CC and Kraft AS (1997) Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits P38 MAPK and stress-activated protein kinase in U937 cells. *J Biol Chem* **272**: 16917–16923.

Furst R, Schroeder T, Eilken HM, Bubik MF, Kiemer AK, Zahler S, and Vollmar AM (2007) MAPK phosphatase-1 represents a novel anti-inflammatory target of glucocorticoids in the human endothelium. *FASEB J* **21**: 74–80.

Giguere V, Hollenberg SM, Rosenfeld MG, and Evans RM (1986) Functional domains of the human glucocorticoid receptor. *Cell* **46**: 645–652.

Gonzales LW, Guttentag SH, Wade KC, Postle AD, and Ballard PL (2002) Differentiation of human pulmonary type II cells in vitro by glucocorticoid plus CAMP. *Am J Physiol Lung Cell Mol Physiol* **283**: L940–L951.

Grunberg K, Sharon RF, Hiltermann TJ, Brahim JJ, Dick EC, Sterk PJ, and Van Krieken JH (2000) Experimental rhinovirus 16 infection increases intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment. *Clin Exp Allergy* **30**: 1015–1023.

Helve O, Pitkanen OM, Andersson S, O'Brodovich H, Kirjavainen T, and Otulakowski G (2004) Low expression of human epithelial sodium channel in airway epithelium of preterm infants with respiratory distress. *Pediatrics* **113**: 1267–1272.

Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, and Evans RM (1985) Primary structure and expression of a functional human glucocorticoid receptor CDNA. *Nature* **318**: 635–641.

Holtzman MJ, Morton JD, Shornick LP, Tyner JW, O'Sullivan MP, Antao A, Lo M, Castro M, and Walter MJ (2002) Immunity, inflammation, and remodeling in the airway epithelial barrier: epithelial-viral-allergic paradigm. *Physiol Rev* **82**: 19–46.

Hoshino M, Nakamura Y, Sim JJ, Yamashiro Y, Uchida K, Hosaka K, and Isogai S (1998) Inhaled corticosteroid reduced lamina reticularis of the basement membrane by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma. *Clin Exp Allergy* **28**: 568–577.

Itani OA, Auerbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB, and Thomas CP (2002) Glucocorticoid-stimulated lung epithelial Na(+) transport is associated with regulated ENaC and Sgk1 expression. *Am J Physiol Lung Cell Mol Physiol* **282**: L631–L641.

Ito K, Barnes PJ, and Adcock IM (2000) Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Mol Cell Biol* **20**: 6891–6903.

Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, and Adcock IM (2006) Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J Exp Med* **203**: 7–13.

Jame AJ, Lackie PM, Cazaly AM, Sayers I, Penrose JF, Holgate ST, and Sampson AP (2007) Human bronchial epithelial cells express an active and inducible biosynthetic pathway for leukotrienes B(4) and C(4). *Clin Exp Allergy* **37**: 880–892.

Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H, and Herrlich P (1990) Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62**: 1189–1204.

Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, and Cato AC (2001) Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO J* **20**: 7108–7116.

Kern JA, Lamb RJ, Reed JC, Daniele RP, and Nowell PC (1988) Dexamethasone inhibition of interleukin 1 beta production by human monocytes. Posttranscriptional mechanisms. *J Clin Invest* **81**: 237–244.

King LS, Nielsen S, and Agre P (1996) Aquaporin-1 water channel protein in lung: ontogeny, steroid-induced expression, and distribution in rat. *J Clin Invest* **97**: 2183–2191.

Kleinert H, Euchenhofer C, Ihrig-Biedert I, and Forstermann U (1996) Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-kappa B. *Mol Pharmacol* **49**: 15–21.

Konig H, Ponta H, Rahmsdorf HJ, and Herrlich P (1992) Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo. *EMBO J* **11**: 2241–2246.

Korhonen R, Lahti A, Hamalainen M, Kankaanranta H, and Moilanen E (2002) Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing MRNA in lipopolysaccharide-treated macrophages. *Mol Pharmacol* **62**: 698–704.

Kwon OJ, Au BT, Collins PD, Baraniuk JN, Adcock IM, Chung KF, and Barnes PJ (1994) Inhibition of interleukin-8 expression by dexamethasone in human cultured airway epithelial cells. *Immunology* **81**: 389–394.

Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, and Blackshear PJ (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha MRNA. *Mol Cell Biol* **19**: 4311–4323.

Lasa M, Mahtani KR, Finch A, Brewer G, Saklatvala J, and Clark AR (2000) Regulation of cyclooxygenase 2 MRNA stability by the mitogen-activated protein kinase P38 signaling cascade. *Mol Cell Biol* **20**: 4265–4274.

Lasa M, Brook M, Saklatvala J, and Clark AR (2001) Dexamethasone destabilizes cyclooxygenase 2 MRNA by inhibiting mitogen-activated protein kinase P38. *Mol Cell Biol* **21**: 771–780.

Lasa M, Abraham SM, Boucheron C, Saklatvala J, and Clark AR (2002) Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK P38. *Mol Cell Biol* **22**: 7802–7811.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, and Landvatter SW (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**: 739–746.

Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, and Allison AC (1988) Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta MRNA. *Proc Natl Acad Sci U S A* **85**: 1204–1208.

Levine SJ, Larivee P, Logun C, Angus CW, and Shelhamer JH (1993) Corticosteroids differentially regulate secretion of IL-6, IL-8, and G-CSF by a human bronchial epithelial cell line. *Am J Physiol* **265**: L360–L368.

Lilly CM, Nakamura H, Kesselman H, Nagler-Anderson C, Asano K, Garcia-Zepeda EA, Rothenberg ME, Drazen JM, and Luster AD (1997) Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J Clin Invest* **99**: 1767–1773.

Liu Y, Gorospe M, Yang C, and Holbrook NJ (1995) Role of mitogen-activated protein kinase phosphatase during the cellular response to genotoxic stress. Inhibition of C-Jun N-terminal kinase activity and AP-1-dependent gene activation. *J Biol Chem* **270**: 8377–8380.

Losel R, andWehling M. (2003) Nongenomic actions of steroid hormones *Nat. Rev. Mol. Cell. Biol*. **4**: 46–55.

Losel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K, and Wehling M (2003) Nongenomic steroid action: controversies, questions, and answers. *Physiol Rev* **83**: 965–1016.

Lu W, Lillehoj EP, and Kim KC (2005) Effects of dexamethasone on Muc5ac mucin production by primary airway goblet cells. *Am J Physiol Lung Cell Mol Physiol* **288**: L52–L60.

Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, Hochberg RB, McKay L, Renoir JM, Weigel NL, Wilson EM, McDonnell DP, and Cidlowski JA (2006) International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. *Pharmacol Rev* **58**: 782–797.

Luecke HF and Yamamoto KR (2005) The glucocorticoid receptor blocks P-TEFb recruitment by NFkappaB to effect promoter-specific transcriptional repression. *Genes Dev* **19**: 1116–1127.

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, and Sigler PB (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**: 497–505.

Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J, and Clark AR (2001) Mitogen-activated protein kinase P38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha MRNA stability. *Mol Cell Biol* **21**: 6461–6469.

Martin LD, Rochelle LG, Fischer BM, Krunkosky TM, and Adler KB (1997) Airway epithelium as an effector of inflammation: molecular regulation of secondary mediators. *Eur Respir J* **10**: 2139–2146.

Mata J, Marguerat S, and Bahler J (2005) Post-transcriptional control of gene expression: a genome-wide perspective. *Trends Biochem Sci* **30**: 506–514.

Matthay MA, Folkesson HG, and Clerici C (2002) Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol Rev* **82**: 569–600.

McKay LI and Cidlowski JA (2000) CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. *Mol Endocrinol* **14**: 1222–1234.

Mills PR, Davies RJ, and Devalia JL (1999) Airway epithelial cells, cytokines, and pollutants. *Am J Respir Crit Care Med* **160**: S38–S43.

Mitchell JA, Belvisi MG, Akarasereenont P, Robbins RA, Kwon OJ, Croxtall J, Barnes PJ, and Vane JR (1994) Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br J Pharmacol* **113**: 1008–1014.

Mittelstadt PR and Ashwell JD (2001) Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J Biol Chem* **276**: 29603–29610.

Mozo L, Gayo A, Suarez A, Rivas D, Zamorano J, and Gutierrez C (1998) Glucocorticoids inhibit IL-4 and mitogen-induced IL-4R alpha chain expression by different posttranscriptional mechanisms. *J Allergy Clin Immunol* **102**: 968–976.

Mukaida N, Morita M, Ishikawa Y, Rice N, Okamoto S, Kasahara T, and Matsushima K (1994) Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* **269**: 13289–13295.

Newton R (2000) Molecular mechanisms of glucocorticoid action: what is important? *Thorax* **55**: 603–613.

Newton R and Holden N (2003) Inhibitors of P38 mitogen-activated protein kinase: potential as anti-inflammatory agents in asthma? *BioDrugs* **17**: 113–129.

Newton R, Hart LA, Stevens DA, Bergmann M, Donnelly LE, Adcock IM, and Barnes PJ (1998a) Effect of dexamethasone on interleukin-1beta-(IL-1beta)-induced nuclear factor-kappaB (NF-kappaB) and kappaB-dependent transcription in epithelial cells. *Eur J Biochem* **254**: 81–89.

Newton R, Seybold J, Kuitert LM, Bergmann M, and Barnes PJ (1998b) Repression of cyclooxygenase-2 and prostaglandin E2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated MRNA. *J Biol Chem* **273**: 32312–32321.

Newton R, Cambridge L, Hart LA, Stevens DA, Lindsay MA, and Barnes PJ (2000) The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1beta-dependent PGE(2) release via mechanistically distinct processes. *Br J Pharmacol* **130**: 1353–1361.

Newton R, Eddleston J, Haddad E, Hawisa S, Mak J, Lim S, Fox AJ, Donnelly LE, and Chung KF (2002) Regulation of kinin receptors in airway epithelial cells by inflammatory cytokines and dexamethasone. *Eur J Pharmacol* **441**: 193–202.

Nissen RM and Yamamoto KR (2000) The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* **14**: 2314–2329.

Ohtsuka T, Kubota A, Hirano T, Watanabe K, Yoshida H, Tsurufuji M, Iizuka Y, Konishi K, and Tsurufuji S (1996) Glucocorticoid-mediated gene suppression of rat cytokine-induced neutrophil chemoattractant CINC/Gro, a member of the interleukin-8 family, through impairment of NF-kappa B activation. *J Biol Chem* **271**: 1651–1659.

Otulakowski G, Rafii B, Harris M, and O'Brodovich H (2006) Oxygen and glucocorticoids modulate alphaENaC MRNA translation in fetal distal lung epithelium. *Am J Respir Cell Mol Biol* **34**: 204–212.

Paine R, III, Rolfe MW, Standiford TJ, Burdick MD, Rollins BJ, and Strieter RM (1993) MCP-1 expression by rat type II alveolar epithelial cells in primary culture. *J Immunol* **150**: 4561–4570.

Pearce D and Kleyman TR (2007) Salt, sodium channels, and SGK1. *J Clin Invest* **117**: 592–595.

Peppel K, Vinci JM, and Baglioni C (1991) The AU-rich sequences in the 3′ untranslated region mediate the increased turnover of interferon MRNA induced by glucocorticoids. *J Exp Med* **173**: 349–355.

Pratt WB, Galigniana MD, Harrell JM, and DeFranco DB (2004) Role of Hsp90 and the Hsp90-binding immunophilins in signalling protein movement. *Cell Signal* **16**: 857–872.

Proud D and Chow CW (2006) Role of viral infections in asthma and chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **35**: 513–518.

Qiu J, Wang CG, Huang XY, and Chen YZ (2003) Nongenomic mechanism of glucocorticoid inhibition of bradykinin-induced calcium influx in PC12 cells: possible involvement of protein kinase C. *Life Sci* **72**: 2533–2542.

Ray A and Prefontaine KE (1994) Physical association and functional antagonism between the P65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* **91**: 752–756.

Ray KP, Farrow S, Daly M, Talabot F, and Searle N (1997) Induction of the E-selectin promoter by interleukin 1 and tumour necrosis factor alpha, and inhibition by glucocorticoids. *Biochem J* **328**: 707–715.

Rhen T and Cidlowski JA (2005) Antiinflammatory action of glucocorticoids – new mechanisms for old drugs. *N Engl J Med* **353**: 1711–1723.

Ristimaki A, Narko K, and Hla T (1996) Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation. *Biochem J* **318**: 325–331.

Rogatsky I, Zarember KA, and Yamamoto KR (2001) Factor recruitment and TIF2/GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. *EMBO J* **20**: 6071–6083.

Rogatsky I, Luecke HF, Leitman DC, and Yamamoto KR (2002) Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc Natl Acad Sci U S A* **99**: 16701–16706.

Rogers DF (2004) Airway mucus hypersecretion in asthma: an undervalued pathology? *Curr Opin Pharmacol* **4**: 241–250.

Saunders MA, Belvisi MG, Cirino G, Barnes PJ, Warner TD, and Mitchell JA (1999) Mechanisms of prostaglandin E2 release by intact cells expressing cyclooxygenase-2: evidence for a 'two-component' model. *J Pharmacol Exp Ther* **288**: 1101–1106.

Schacke H, Docke WD, and Asadullah K (2002) Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* **96**: 23–43.

Scheinman RI, Cogswell PC, Lofquist AK, and Baldwin AS, Jr. (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* **270**: 283–286.

Schleimer RP (1993) An overview of glucocorticoid anti-inflammatory actions. *Eur J Clin Pharmacol* **45** (Suppl 1): S3–S7.

Schleimer RP (2004) Glucocorticoids suppress inflammation but spare innate immune responses in airway epithelium. *Proc Am Thorac Soc* **1**: 222–230.

Sheppard KA, Phelps KM, Williams AJ, Thanos D, Glass CK, Rosenfeld MG, Gerritsen ME, and Collins T (1998) Nuclear integration of glucocorticoid receptor and nuclear factor-kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. *J Biol Chem* **273**: 29291–29294.

Slack DN, Seternes OM, Gabrielsen M, and Keyse SM (2001) Distinct binding determinants for ERK2/P38alpha and JNK map kinases mediate catalytic activation and substrate selectivity of map kinase phosphatase-1. *J Biol Chem* **276**: 16491–16500.

Smoak K and Cidlowski JA (2006) Glucocorticoids regulate tristetraprolin synthesis and post-transcriptionally regulate tumor necrosis factor alpha inflammatory signaling. *Mol Cell Biol* **26**: 9126–9135.

Staples KJ, Bergmann MW, Barnes PJ, and Newton R (2003) Evidence for post-transcriptional regulation of interleukin-5 by dexamethasone. *Immunology* **109**: 527–535.

Stellato C (2004) Post-transcriptional and nongenomic effects of glucocorticoids. *Proc Am Thorac Soc* **1**: 255–263.

Stellato C, Beck LA, Gorgone GA, Proud D, Schall TJ, Ono SJ, Lichtenstein LM, and Schleimer RP (1995) Expression of the chemokine RANTES by a human bronchial epithelial cell line. modulation by cytokines and glucocorticoids. *J Immunol* **155**: 410–418.

Stellato C, Matsukura S, Fal A, White J, Beck LA, Proud D, and Schleimer RP (1999) Differential regulation of epithelial-derived C-C chemokine expression by IL-4 and the glucocorticoid budesonide. *J Immunol* **163**: 5624–5632.

Suzuki T, Yamaya M, Sekizawa K, Yamada N, Nakayama K, Ishizuka S, Kamanaka M, Morimoto T, Numazaki Y, and Sasaki H (2000) Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **278**: L560–L571.

Swantek JL, Cobb MH, and Geppert TD (1997) Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol Cell Biol* **17**: 6274–6282.

Szapary D, Huang Y, and Simons SS, Jr. (1999) Opposing effects of corepressor and coactivators in determining the dose–response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated gene expression. *Mol Endocrinol* **13**: 2108–2121.

Tanaka M, Inase N, Fushimi K, Ishibashi K, Ichioka M, Sasaki S, and Marumo F (1997) Induction of aquaporin 3 by corticosteroid in a human airway epithelial cell line. *Am J Physiol* **273**: L1090–L1095.

Tang ML, Wilson JW, Stewart AG, and Royce SG (2006) Airway remodelling in asthma: current understanding and implications for future therapies. *Pharmacol Ther* **112**: 474–488.

Tasker JG, Di S, and Malcher-Lopes R (2006) Minireview: rapid glucocorticoid signaling via membrane-associated receptors. *Endocrinology* **147**: 5549–5556.

Thoren S and Jakobsson PJ (2000) Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. *Eur J Biochem* **267**: 6428–6434.

Tillmann HC, Stuck BA, Feuring M, Rossol-Haseroth K, Tran BM, Losel R, Schmidt BM, Hormann K, Wehling M, and Schultz A (2004) Delayed genomic and acute nongenomic action of glucocorticosteroids in seasonal allergic rhinitis. *Eur J Clin Invest* **34**: 67–73.

Tobler A, Meier R, Seitz M, Dewald B, Baggiolini M, and Fey MF (1992) Glucocorticoids downregulate gene expression of GM-CSF, NAP-1/IL-8, and IL-6, but not of M-CSF in human fibroblasts. *Blood* **79**: 45–51.

Towne JE, Harrod KS, Krane CM, and Menon AG (2000) Decreased expression of aquaporin (AQP)1 and AQP5 in mouse lung after acute viral infection. *Am J Respir Cell Mol Biol* **22**: 34–44.

Urbach V, Walsh DE, Mainprice B, Bousquet J, and Harvey BJ (2002) Rapid non-genomic inhibition of ATP-induced Cl-secretion by dexamethasone in human bronchial epithelium. *J Physiol* **545**: 869–878.

Urbach V, Verriere V, Grumbach Y, Bousquet J, and Harvey BJ (2006) Rapid anti-secretory effects of glucocorticoids in human airway epithelium. *Steroids* **71**: 323–328.

van de Stolpe A, Caldenhoven E, Raaijmakers JA, van der Saag PT, and Koenderman L (1993) Glucocorticoid-mediated repression of intercellular adhesion molecule-1 expression in human monocytic and bronchial epithelial cell lines. *Am J Respir Cell Mol Biol* **8**: 340–347.

van de Stolpe A, Caldenhoven E, Stade BG, Koenderman L, Raaijmakers JA, Johnson JP, and van der Saag PT (1994) 12-O-Tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. *J Biol Chem* **269**: 6185–6192.

Verriere VA, Hynes D, Faherty S, Devaney J, Bousquet J, Harvey BJ, and Urbach V (2005) Rapid effects of dexamethasone on intracellular PH and Na + /H+ exchanger activity in human bronchial epithelial cells. *J Biol Chem* **280**: 35807–35814.

Volonaki E, Psarras S, Xepapadaki P, Psomali D, Gourgiotis D, and Papadopoulos NG (2006) Synergistic effects of fluticasone propionate and salmeterol on inhibiting rhinovirus-induced epithelial production of remodelling-associated growth factors. *Clin Exp Allergy* **36**: 1268–1273.

Wade KC, Guttentag SH, Gonzales LW, Maschhoff KL, Gonzales J, Kolla V, Singhal S, and Ballard PL (2006) Gene induction during differentiation of human pulmonary type II cells in vitro. *Am J Respir Cell Mol Biol* **34**: 727–737.

Wang JH, Trigg CJ, Devalia JL, Jordan S, and Davies RJ (1994) Effect of inhaled beclomethasone dipropionate on expression of proinflammatory cytokines and activated eosinophils in the bronchial epithelium of patients with mild asthma. *J Allergy Clin Immunol* **94**: 1025–1034.

Wang J, Zhu Z, Nolfo R, and Elias JA (1999) Dexamethasone regulation of lung epithelial cell and fibroblast interleukin-11 production. *Am J Physiol* **276**: L175–L185.

Wang Q, Blackford JA, Jr., Song LN, Huang Y, Cho S, and Simons SS, Jr. (2004) Equilibrium interactions of corepressors and coactivators with agonist and antagonist complexes of glucocorticoid receptors. *Mol Endocrinol* **18**: 1376–1395.

Wesselborg S, Bauer MK, Vogt M, Schmitz ML, and Schulze-Osthoff K (1997) Activation of transcription factor NF-kappaB and P38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. *J Biol Chem* **272**: 12422–12429.

Winzen R, Kracht M, Ritter B, Wilhelm A, Chen CY, Shyu AB, Muller M, Gaestel M, Resch K, and Holtmann H (1999) The P38 MAP kinase pathway signals for cytokine-induced MRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J* **18**: 4969–4980.

Wissink S, van Heerde EC, vand der BB, and van der Saag PT (1998) A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. *Mol Endocrinol* **12**: 355–363.

Wu JJ, Zhang L, and Bennett AM (2005) The noncatalytic amino terminus of mitogen-activated protein kinase phosphatase 1 directs nuclear targeting and serum response element transcriptional regulation. *Mol Cell Biol* **25**: 4792–4803.

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, and Karin M (1990) Transcriptional interference between C-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction. *Cell* **62**: 1205–1215.

Zhou J, Kang ZM, Xie QM, Liu C, Lou SJ, Chen YZ, and Jiang CL (2003) Rapid nongenomic effects of glucocorticoids on allergic asthma reaction in the guinea pig. *J Endocrinol* **177**: R1–R4.

19

The Airway Epithelium as a Target for the Therapeutic Actions of β_2-Adrenoceptor Agonists and Muscarinic-receptor Antagonists

Mark A. Giembycz
Department of Pharmacology & Therapeutics, Institute of Infection, Immunity and Inflammation, Faculty of Medicine, University of Calgary, Alberta, Canada

19.1 Introduction

The topical application of drugs for the treatment of airway inflammatory disorders such as asthma and chronic obstructive pulmonary disease (COPD) is a widely adopted method of administration as it directly targets the organ of interest and, theoretically, should be associated with a reduced adverse-events profile over drugs given orally, where marked systemic exposure can be problematic. An additional advantage is that drugs given by inhalation will first come into contact with the airway epithelium, for which profound pro-inflammatory roles are now recognized (Barnes, 1996; Schwiebert *et al*., 1996). The ability of airway epithelia to elaborate lipids, chemokines, cytokines and pro-fibrotic mediators, makes this tissue a primary and critical target for the anti-inflammatory actions of inhaled corticosteroids (ICS) (Barnes, 1996). Moreover, the airway epithelium must also be considered an important target for the activity of inhaled long-acting β_2-adrenoceptor agonists (LABAs) as these drugs, in some way, enhance the clinical efficacy of ICSs to a level that cannot be achieved by the corticosteroid alone. Emerging evidence indicates that LABAs probably achieve this effect by augmenting the efficacy of ICSs at the level of airway epithelial cells (and other target tissues) rather than eliciting mechanistically-distinct, complementary anti-inflammatory activity. The importance of the epithelium as a site of drug action may also extend to muscarinic receptor antagonists (also known as anticholinergics). Indeed, acetylcholine (ACh) is produced by, and can also activate, airway epithelia to release mediators of inflammation that may have relevance to the pathophysiology of COPD.

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

The primary purpose of this chapter is to detail the structure and function of β_2-adrenoceptors expressed by human airway epithelial cells and how targeting this receptor with LABAs enhances corticosteroid action. The possibility that agonism of β_2-adrenoceptors on epithelial cells can also regulate tone of the underlying airway smooth muscle is also discussed. Finally, it has been proposed that muscarinic receptor antagonists possess anti-inflammatory activity in chronic airway diseases unrelated to their ability to promote bronchodilatation. This is a novel and highly attractive idea and is discussed here in respect of airway epithelial cells.

19.2 The β_2-adrenoceptor as a therapeutic target

19.2.1 β_2-Adrenoceptors on human airway epithelial cells

The human β_2-adrenoceptor gene is located on the long arm of chromosome 5 and codes for an intronless, 1200 bp product of 413 amino acids (46.5 kDa) that responds to the endogenous hormones adrenaline and noradrenaline. Relative to most other structural, resident and pro-inflammatory cells within the lung, airway epithelial cells express a relatively high density of functional β_2-adrenoceptors (Penn *et al*., 1994), which has been estimated at 7000–9000 sites/cell (Kelsen *et al*., 1995; Penn *et al*., 1994). Similar levels are also found on a number of human airway epithelial cells lines including BEAS-2B (Kelsen *et al*., 1997a, 1997b), 16HBE14o- and Calu-3 (Abraham *et al*., 2004). Only human airway smooth muscle (HASM) cells are believed to express a significantly higher number of β_2-adrenoceptors (30 000–40 000 sites/cell) (Johnson, 2002).

19.2.2 Functional effects of β_2-adrenoceptor agonists human airway epithelial cells

Although β_2-adrenoceptor agonists given as a monotherapy are not anti-inflammatory, they do elicit a number of non-bronchodilator actions on pro-inflammatory and immune cells (structural, infiltrated and resident) that may be of some clinical benefit. There is reasonable evidence from in vitro and in vivo studies that β_2-adrenoceptor agonists increase ciliary beat frequency (Devalia *et al*., 1992; Lansley *et al*., 1992; Nishimura *et al*., 2002; Sanderson and Dirksen, 1989; Verdugo *et al*., 1980; Yang *et al*., 1996), which presumably accounts for the ability of these drugs to improve mucociliary transport (Foster *et al*., 1976; Sackner *et al*., 1976; Yeates *et al*., 1986). Hastie *et al*. (1997) have also found that the short-acting β_2-adrenoceptor agonist, salbutamol, upregulates in vivo the expression of stress proteins in human airway epithelial cells including heat shock protein (HSP)-72 and HSP-73. This finding also may be of therapeutic relevance given that the induction of a stress response can protect against a secondary insult (Mizzen and Welch, 1988). Similarly, the suggested deleterious ability of corticosteroids to induce apoptosis of human airway epithelial cells is blocked by short-acting and LABAs (salbutamol and formoterol respectively) (Tse *et al*., 2003).

The inhibitory activity of β_2-adrenoceptors agonists on responses that, classically, are considered to be pro-inflammatory has not been studied in great detail. However, procaterol, a potent, short-acting compound, is reported to suppress the release, from BEAS-2B cells, of several interleukin (IL)-1β/tumour necrosis factor-α (TNFα)-induced cytokines/chemokines including regulated upon activation, normal T-cell-expressed and secreted (RANTES), granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-8 (Koyama *et al*.,

1999). Similar data were obtained using primary human airway epithelial cells with the LABAs formoterol (Korn *et al*., 2001) and salmeterol (Sabatini *et al*., 2003), although those results were not confirmed in another study with salbutamol using cells harvested from both normal and asthmatic subjects (Gormand *et al*., 1995). β_2-adrenoceptor agonists also have been found to attenuate the upregulation of certain adhesion molecules including intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Oddera *et al*., 1998; Sabatini *et al*., 2003).

In contrast, several potentially adverse effects of β_2-adrenoceptor agonists on airway epithelial cell function have been documented. In particular, treatment of BEAS-2B and 16-HBE14o- cells with salbutamol enhanced cell proliferation (Nishimura *et al*., 2002) implying that regular administration of this class of drugs as a monotherapy could, theoretically, contribute to airway remodelling that characterizes both asthma and COPD.

19.2.3 Airway epithelial cell β_2-adrenoceptors and the regulation of airway smooth muscle tone

Elegant studies by Liggett and his colleagues (McGraw *et al*., 2000) have revealed new insights in to the regulation of airway smooth function by β_2-adrenoceptors expressed on airway epithelial cells. Specifically, the investigators have discovered that the density of β_2-adrenoceptors on epithelial cells has an impact on the tone of the underlying smooth muscle. Thus, using the rat Clara cell secretory protein promoter, which resulted in targeted overexpression (twofold) in mice of epithelial cell β_2-adrenoceptor number, McGraw *et al*. (2000) found that the dose of methacholine (MCh) required to increase, over baseline, airway resistance by 200 per cent was significantly higher when compared to non-transgenic littermates. The protection afforded against MCh-induced bronchoconstriction in the transgenic animals was the same as that produced by salbutamol given by inhalation to mice not expressing the transgene (McGraw *et al*., 2000). These data are consistent with a multi-state model of G-protein-coupled receptors where the effector, adenylyl cyclase in this case, is activated by the receptor in the *absence* of agonist. Obviously, to account for this model it is necessary to propose that the equilibrium in the absence of agonist normally favours the inactive conformation. In the experiments described by McGraw *et al*. (2000) a twofold overexpression of β_2-adrenoceptors on the airway epithelium allowed sufficient spontaneous coupling to severely limit MCh-induced tone in the absence of agonist. The mechanism underlying this protective effect is unknown, but it is not apparently due to the enhanced release from the epithelium of nitric oxide or prostaglandin E_2 (McGraw *et al*., 2000). Assuming the pharmacological behaviour of the murine β_2-adrenoceptor can be extrapolated to humans, these transgenic animals exhibit, what may be described as, an anti-asthma and/or anti-COPD phenotype. These data thus tempt speculation that targeted overexpression of β_2-adrenoceptors to airway epithelial cells could provide a genetic therapy for airway inflammatory diseases where bronchodilatation is a desired therapeutic outcome.

19.2.4 Enhancement of ICS action by LABAs

The critical clinical observation

In 1994, Greening and colleagues conducted a double-blind, parallel group trial of 6 months duration in 426 asthmatic subjects who were symptomatic despite maintenance therapy with

the ICS, beclomethasone dipropionate (BDP; 200 μg b.i.d.). Subjects were randomized to receive either salmeterol xinafoate (50 μg b.i.d.) and BDP (200 μg b.i.d.; $n = 220$) delivered via separate inhaler devices, or BDP alone at a higher dose of 500 μg (b.i.d.; $n = 206$). Both treatment options significantly improved lung function (mean morning peak expiratory flow rate (PEFR)), but the LABA/ICS combination therapy was superior at all time points. Other endpoints that favoured salmeterol/BDP over high-dose BDP alone included diurnal variation in PEFR, daytime and night-time symptoms, and rescue bronchodilator consumption. Significantly, there was no significant difference between the two treatment groups in exacerbation rate indicating that salmeterol, given chronically with BDP, was not associated with any risk of asthma deterioration over the duration of the study. Thus, the addition of salmeterol to a standard dose of BDP was more effective clinically than increasing, by 250 per cent, the dose of BDP (Greening *et al*., 1994).

Confirmation of effect

The superior clinical benefit of salmeterol and BDP given in combination was confirmed subsequently in more severe subjects in whom asthma was not controlled on BDP (500 μg b.i.d.) or equivalent (Woolcock *et al*., 1996). In this double-blind, parallel group study of 6 months' duration 738 subjects at 72 centres in Australia were randomized to receive either salmeterol (50 or 100 μg b.i.d.) in combination with BDP (500 μg b.i.d.), delivered by separate inhaler devices, or BDP alone at a higher dose (1000 μg b.i.d.). Consistent with the results of Greening *et al*. (1994), subjects taking either dose of salmeterol showed mean improvements of >45 and >30 L/min in their morning and evening PEFR respectively, which was markedly superior to that achieved in individuals on the higher dose of BDP only (PEFR morning: 16 L/min; PEFR evening 6 L/min). Moreover, rescue bronchodilator use and symptoms in those subjects taking either dose of salmeterol were significantly lower when compared to individuals in the BDP (1000 μg b.i.d.) treatment group. There was no significant difference in the clinical benefit afforded by the two doses of salmeterol suggesting that the dose of 100 μg (b.i.d.) is supra-maximal. Exacerbation rates did not differ among the three treatment groups confirming, again, that there was no deterioration in asthma control in those individuals taking the combination therapy. Thus, the addition of salmeterol to BDP was more effective clinically than doubling the dose of BDP.

Superiority of LABA/ICS combination therapy is class-specific

Since the seminal report of Greening *et al*. (1994), many trials have been conducted comparing the clinical effectiveness in asthma of LABA/ICS combination therapies with a higher-dose of an ICS alone. What has emerged, unambiguously, is that the clinical superiority of salmeterol and BDP in combination over higher dose ICS alone is unequivocal and class-specific (i.e. it is not peculiar to salmeterol or BDP, but a generic effect of LABAs and ICSs when used in combination). For example, in a multi-centre, double-blind, parallel group study of 6 months' duration involving 496 symptomatic asthmatic patients with a history of exacerbations on ICS (500–800 μg b.i.d.), Ind *et al*. (2003) demonstrated that adding salmeterol (50 μg b.i.d.) to another ICS, fluticasone propionate (250 μg b.i.d.), was clinically superior to doubling the dose of that corticosteroid. Thus, the salmeterol/fluticasone combination significantly improved mean morning PEFR by 42.1 L/min, which was more than twice the improvement achieved with fluticasone given as a monotherapy at either 250 μg

(b.i.d.) or 500 μg (b.i.d.). Similar data in favour of the combination therapy were obtained when symptoms and exacerbation rate were used as endpoints (Ind *et al*., 2003).

In addition, the landmark FACET (Formoterol And Corticosteroids Establishing Therapy) study also confirmed the superiority of the combination therapy when exacerbation rate was used as the primary outcome measure. Indeed, the exacerbation rate in subjects with moderately severe asthma was lower when the LABA, formoterol (9 μg b.i.d.,) was added to low and high dose of another corticosteroid, budesonide (i.e. 100 μg or 400 μg b.i.d.) when compared to the ICS alone (Pauwels *et al*., 1997). The clinical superiority of LABA/ICS combination therapies also extends to subjects with mild persistent asthma in whom the optimal treatment regime is uncertain. Thus, the OPTIMA (Oxis and Pulmicort Turbuhaler In the Management of Asthma) study convincingly demonstrated that adding formoterol (4.5 μg b.i.d.), to low-dose budesonide (100 μg b.i.d.) for 1 year in subjects with mild asthma was more effective than doubling the dose of ICS in increasing the time to first severe asthma exacerbation (O'Byrne *et al*., 2001).

Implications for treatment

Compelling evidence now supports the concept that the addition of a LABA to a regular ICS is more effective at improving asthma control, lung function and reducing exacerbation frequency than increasing, even quadrupling, the dose of ICS (Barnes, 2001a; Greening *et al*., 1994; Kankaanranta *et al*., 2004; O'Byrne *et al*., 2001, 2005; Pauwels *et al*., 1997; Shrewsbury *et al*., 2000; Woolcock *et al*., 1996). Based upon the results of a large number of clinical trials, the recently updated GINA (Global INitiative for Asthma) guidelines now recommend combination therapies for asthma where symptoms are not adequately controlled on low-dose ICS. Moreover, the results of the TORCH (TOwards a Revolution in COPD Health) study published in February 2007 in the *New England Journal of Medicine* (Calverley *et al*., 2007) found that the combination of fluticasone and salmeterol significantly improved lung function and health status, and reduced exacerbation rate and risk of death in patients with COPD. Significantly, these effects were substantially superior to the clinical efficacy of either agent given alone as a monotherapy (Calverley *et al*., 2007). It is likely, therefore, that current international guidelines will soon be amended, endorsing the use of LABA/ICS combinations for the treatment of moderate/severe COPD.

19.2.5 LABA/ICS interactions: additivity or synergy?

Despite the therapeutic advantages of LABA/ICS 'combination' therapies, the mechanistic basis for their superior efficacy remains vague. According to traditional dogma, LABAs bind to cell surface β_2-adrenoceptors and augment the activity of one or more isoforms of adenylyl cyclase by a Gsα-dependent mechanism. This catalysis increases the intracellular concentration of cyclic adenosine-3′, 5′-monophosphate (cAMP) and activates cAMP-dependent protein kinase (PKA) with an ultimate functional consequence such as bronchodilatation (Figure 19.1) (Giembycz and Newton, 2006).

In the context of this classical pathway, there is good evidence that ICSs improve β_2-adrenoceptor-mediated signalling in the lung. Indeed, corticosteroids increase β_2-adrenoceptor density (Mak *et al*., 1995a, 1995b), reduce functional desensitization of the receptor (Chong *et al*., 1997) and enhance both Gsα expression and coupling to adenylyl cyclase (Kalavantavanich and Schramm, 2000). However, the mechanism by which LABAs

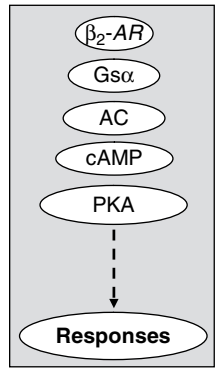


Figure 19.1 Classical β_2-adrenoceptor (β_2-AR) signalling in which the β_2-AR couples via Gsα to adenylyl cyclase (AC), increases the formation of cAMP and activates PKA to elicit responses such as smooth muscle relaxation

enhance GR-dependent signalling is largely unexplored. It has been shown that budesonide can suppress TNF-α-induced GM-CSF release from human airway epithelial cells by a mechanism that is enhanced by formoterol (Korn *et al*., 2001). Similar effects have been reported with salmeterol on the inhibition, by fluticasone, of both IL-8 and eotaxin release from HASM cells (Pang and Knox, 2000, 2001). These general findings have been extended to studies in which viruses have been used as stimuli, to assess the potential efficacy of ICS/LABA combination therapies in exacerbations of asthma and COPD. Thus, exposure of BEAS-2B cells to rhinovirus resulted in the elaboration of two factors putatively involved in airway remodelling (vascular endothelial growth factor, fibroblast growth factor-2) by a mechanism that was inhibited by both fluticasone and, to a lesser extent, salmeterol. Significantly, these two drugs acted synergistically when used in combination (Volonaki *et al*., 2006). Fluticasone and salmeterol have also been shown to inhibit, in an additive or even synergistic manner, rhinovirus-induced chemokine (CXCL5, CXCL8, CXCL10, CCL5) release from BEAS-2B cells (Edwards *et al*., 2006) and this efficacy seemingly can be extrapolated to murine in vivo systems (Singam *et al*., 2006). Indeed, the airway hyper-responsiveness and inflammation in sensitized mice exposed to respiratory syncytial virus is more effectively suppressed by the combination of salmeterol and fluticasone than either drug alone. While, collectively, these studies demonstrate that a corticosteroid and a LABA in combination suppress pro-inflammatory cytokine production in an additive or, possibly, synergistic manner, little information has been published on the underlying molecular mechanism(s). At least two plausible theories that are not mutually exclusive may account for the clinical superiority of LABA/ICS combination therapies over ICSs alone:

1. LABAs and ICSs activate mechanistically-distinct pathways that combine to produce an additive response.
2. LABAs augment the activity of ICSs through a common mechanism(s) to produce a synergistic response.

Evidence for which of these two theories might best account of the enhanced efficacy of LABA/ICS combination therapies comes from clinical studies. Thus, despite the ability

in vitro of LABAs given as a monotherapy to suppress several induces of inflammation (see section above), they do not evoke significant/clinically-relevant anti-inflammatory effects in vivo (Howarth *et al*., 2000; Roberts *et al*., 1999). This fact argues against a major role for separate mechanisms that combine to elicit an additive effect. Conversely, Pauwels *et al*. (1997) reporting for the FACET International Study Group, found that formoterol reduced exacerbation rate and asthma severity in patients taking inhaled budesonide to a greater degree than those subjects who received the same dose of budesonide as a single medication. Those data suggest that, contrary to masking the underlying inflammation (a concern when β_2-adrenoceptor agonists are administered chronically as a monotherapy (reviewed in Sears and Taylor (1994))), LABAs enhance the clinical efficacy of ICSs to a level that cannot be achieved by the ICS alone. Moreover, in COPD, persuasive evidence is available that the superiority of the combination therapy is due to enhanced anti-inflammatory activity (Barnes *et al*., 2006). Thus, while ICSs given as a monotherapy to subjects with COPD do not reduce key pro-inflammatory cell numbers resident within the lung (Gizycki *et al*., 2002; Hattotuwa *et al*., 2002; Keatings *et al*., 1996), a significant widespread anti-inflammatory activity has been noted with a LABA/ICS combination therapy in both airway biopsies and in induced sputum (Barnes *et al*., 2006). Thus, these data favour an interpretation consistent with a positive interaction (synergy) between LABAs and ICSs.

19.2.6 Modelling the enhancement of ICS action by LABAs

Repression of inflammatory gene expression by ICSs is believed to occur by at least two general mechanisms that are not mutually exclusive. The classical mode of corticosteroid action is termed *trans*repression in which the activity of key *pro-inflammatory* transcription factors, such as nuclear factor (NF)-κB and activator protein (AP)-1, is inhibited (Barnes, 2001b). However, in simple systems corticosteroids have been shown to be relatively weak inhibitors of NF-κB- and AP-1-dependent transcription (Chivers *et al*., 2004) implying that additional processes must be operative to account for their anti-inflammatory effect. In this respect, a less well documented mechanism that, paradoxically, might be more clinically relevant is the induction (*trans*activation) by corticosteroids of *anti-inflammatory* genes, which then repress pro-inflammatory processes (Clark, 2003; Lasa *et al*., 2002; Newton *et al*., 1998; Newton, 2000).

The ability of LABAs to enhance the *trans*activation of anti-inflammatory genes in human airway epithelial cells has been investigated in some detail. In one study, Kaur and colleagues (Kaur *et al*., 2008) modelled this response utilizing a classical glucocorticoid response element (GRE)-dependent luciferase reporter construct stably transfected in to BEAS-2B cells, which contains two tandem copies of a consensus GRE site upstream of a minimal β-globin promoter (Chivers *et al*., 2004). In this simple system, LABAs did not activate the GRE reporter construct, but markedly potentiated corticosteroid-induced transcription. This is illustrated graphically in Figure 19.2. Thus, dexamethasone activated the GRE-dependent reporter construct in a concentration-dependent manner with a mean EC_{50} of 26.2 nM. Salmeterol, at a concentration (100 nM) that maximally activated cAMP-response element-dependent transcription, had no effect on the potency of budesonide (mean $EC_{50} = 20$ nM), but augmented, 2.2-fold, the induction of the luciferase gene at all concentrations of dexamethasone above 3 nM. Qualitatively identical data were obtained with other LABA/ICS combinations including salmeterol/fluticasone, salmeterol/budesonide, formoterol/fluticasone, formoterol/budesonide and formoterol/dexamethasone (Kaur *et al*.,

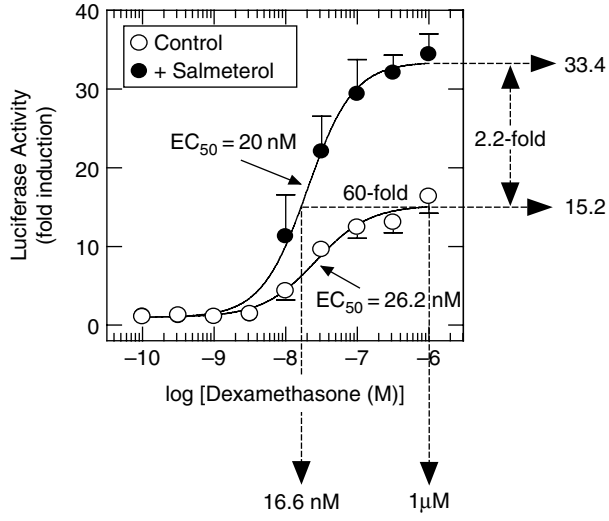


Figure 19.2 Enhancement by salmeterol of dexamethasone-induced action of a GRE reporter construct in BEAS-2B airway epithelial cells. BEAS-2B cells stably harbouring a GRE reporter construct were treated with salmeterol (100 nM) in the absence and presence of dexamethasone (0.1 nM to 1 μM). After 6 h, cells were harvested for luciferase assay. Data are expressed as fold-induction and are plotted as means ± s.e. mean of four independent determinations. See text for further details

2008). These results unequivocally demonstrate that LABAs and corticosteroids can interact synergistically and that this is a class-specific effect.

In addition to enhancing GRE-dependent transcription, formoterol was corticosteroid-sparing in this model (Figure 19.2) (Kaur *et al*., 2008). Thus, dexamethasone, at a concentration of 1 μM, produced a 15.2-fold induction of the luciferase gene. However, in the presence of salmeterol (100 nM), which was inactive, the same degree of gene induction was achieved at a concentration of dexamethasone (2.2 nM) that was 60-fold lower (Figure 19.2). Although outside the remit of this chapter, it is important to state that these effects in BEAS-2B cells were also seen in primary HASM cells harbouring the same reporter construct indicating that the synergistic interaction between LABAs and ICSs was not peculiar to the epithelial cell line and is probably characteristic of all corticosteroid responsive cells that express β_2-adrenoceptors.

Further studies also found that the enhancement by LABAs of corticosteroid-induced gene was abolished by the selective β_2-adrenoceptor antagonist, ICI 118551, and mimicked by forskolin implicating a process that requires β_2-adrenoceptor-mediated activation of adenylyl cyclase. Furthermore, the elevation of cAMP is likely to result in the activation of the classical signalling pathway (Figure 19.1) as the synergy between LABAs and ICSs was lost in cells infected with an adenovirus vector encoding a highly-selective inhibitor of PKA (Kaur *et al*., 2008).

19.2.7 Do LABAs enhance GRE-dependent transcription of 'real' genes?

A crucial detail to establish is whether the synergy between LABAs and corticosteroids obtained with the GRE-reporter construct also occurs for the induction by corticosteroids of

real anti-inflammatory genes. If so, then the process of enhanced *trans*activation described above could explain, at least in part, the therapeutic benefit of LABA/ICS combination therapies in asthma and COPD, especially in those clinical situations where the dose of ICS is close to, or at the top of, the dose–response curve.

Kaur *et al*. (2008) have examined the effect of LABAs on a number of potential, corticosteroid-inducible anti-inflammatory genes expressed by airway epithelial cells to gauge the credibility of the data obtained on the GRE-reporter construct. Two of these are discussed here: glucocorticoid-induced leucine zipper (GILZ), a gene that when induced suppresses the elaboration of pro-inflammatory cytokines from human airway epithelial cells in response to a variety of stimuli (Eddleston *et al*., 2007), and $p57^{kip2}$, a potent, tight-binding inhibitor of several cyclin-dependent kinase complexes involved in G1 and S phase, which negatively regulates cell proliferation (Lee *et al*., 1995). As shown in Figure 19.3(a), exposure of BEAS-2B cells to dexamethasone increased $p57^{kip2}$ mRNA in a time-dependent manner (12.9- and 26.5-fold at 6 h and 18 h respectively) whereas salmeterol had little, if

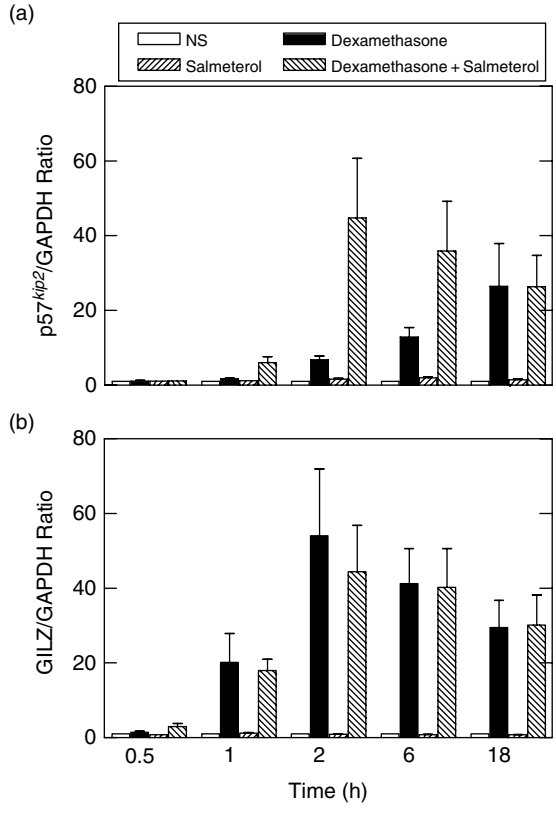


Figure 19.3 Effect of salmeterol on the induction by dexamethasone of GILZ and $p57^{kip2}$. BEAS-2B cells were treated with dexamethasone (1 μM) salmeterol (0.1 μM), a combination of the two drugs or vehicle for 0.5 to 18 h and harvested for real-time PCR analysis of $p57^{kip2}$, GILZ and GAPDH using the SYBR Green^{ER} method. Data are expressed as a ratio to the house-keeping gene GAPDH. See text for further details

any, effect (1.97- and 1.43-fold induction at the same time-points respectively). When cells were exposed to salmeterol and dexamethasone concurrently a marked synergistic induction of the $p57^{kip2}$ gene was evoked at all time-points beyond 0.5 h where mRNA levels were measured. Thus, the effect of dexamethasone and salmeterol on the expression of $p57^{kip2}$, alone and in combination, mirrors very closely the data obtained with the GRE-reporter construct (Figure 19.2). In contrast, while dexamethasone similarly promoted a robust induction of the GILZ gene and salmeterol was inactive, the combination of both drugs elicited an effect that was the same as the corticosteroid alone (Figure 19.3(b)). In this situation, the GILZ gene does not behave in a manner predicted by the GRE reporter. Collectively, these data imply that LABAs are capable of augmenting transcription only of a subset of corticosteroid-sensitive genes and that the degree to which they are able to enhance GRE-dependent transcription will vary from gene to gene.

19.2.8 How do LABAs enhance the action of ICSs?

Arguably, a 'holy grail' of combination therapy research has been to discover the molecular mechanism(s) by which LABAs augment corticosteroid action. LABAs have been shown to enhance the translocation of the glucocorticoid receptor (GR) from the cytosol to the nucleus, even in the *absence* of exogenous corticosteroid (Eickelberg *et al*., 1999; Roth *et al*., 2002, Usmani *et al*., 2005). This, so-called, ligand-independent translocation, which is a well recognized action of cAMP on other steroid hormone receptors (Cenni and Picard, 1999; Weigel and Zhang, 1998), has been reported in a variety of cell types relevant to the treatment of respiratory diseases (Eickelberg *et al*., 1999; Roth *et al*., 2002), including airway epithelial cells (Usmani *et al*., 2005); it has also been observed in vivo in human subjects (Usmani *et al*., 2005). Historically, therefore, it has been suggested that enhanced nuclear translocation of the GR in to the nucleus may account for the superior clinical benefit of LABA/ICS combination therapies. Further support for this assertion is that the combination therapy is associated with increased binding of the GR to GREs on target genes (Korn *et al*., 1998; Miller-Larsson and Selroos, 2006), which could be consistent with the enhanced GR:DNA binding seen in cells overexpressing PKA (Rangarajan *et al*., 1992).

Despite these data, there is evidence from a number of studies that, when considered together, question the overall importance of this mechanism. Reference to Figure 19.2 shows that although salmeterol enhanced GRE-dependent transcription it did not, by itself, activate the reporter construct and had no effect on the potency of dexamethasone (i.e. the EC_{50} values in the absence and presence of salmeterol were not significantly different). This finding supports data obtained from clinical studies where one would predict that the ligand-independent translocation of GR to the nucleus evoked by LABAs would be anti-inflammatory, which it is not (Howarth *et al*., 2000; Roberts *et al*., 1999). In addition, salmeterol enhanced GRE-dependent transcription in the presence of concentrations of dexamethasone that will promote the translocation of all the GR to the nucleus (Figure 19.2) (Chivers *et al*., 2004). This finding is clinically relevant as there are some asthmatic subjects who are not well controlled by high-dose ICS that, arguably, promote the translocation of all available GR to the nucleus. In contrast, asthma control in many of these same individuals is achieved following the administration of a LABA in combination with the same ICS at a lower dose. Indeed, >75 per cent of the GR in airway epithelial cells is found in the nucleus following the administration of a moderate dose of inhaled BDP (800 μg) to asthmatic subjects (Usmani *et al*., 2005). Based on these data, it is difficult to rationalize further GR

translocation as the primary mechanism for the improvement in asthma and COPD control produced by the addition of a LABA, especially in those individuals receiving high-dose ICS. Alternative explanations are required.

One attractive possibility is that LABAs, by virtue of their ability to elevate cAMP, increase the expression of functional GRs. Indeed, pretreatment of human skin fibroblasts with dibutyryl-cAMP increases 2.6-fold the number of specific [^{3}H]dexamethasone binding sites (Oikarinen *et al*., 1984). Comparable data have been derived from rat hepatoma cells treated with 8-Br-cAMP or forskolin in which GR number was significantly increased by a mechanism attributable, at least in part, to GR mRNA stabilization (Dong *et al*., 1989). Of significance is that an increase in GR density is paralleled by enhanced GRE-dependent transcription (Hirst *et al*., 1990; Szapary *et al*., 1996; Zhang *et al*., 2007).

Another highly likely possibility, that is not mutually exclusive with the mechanisms described above, is that LABAs enhance GR-mediated transcription by mechanisms that operate predominantly within the nucleus (i.e. on the transcriptional process itself). This is probably very complex, involving numerous transcriptional co-factors, co-activators, changes in chromatin structure, acetylation, methylation and/or other modifications that impact on transcription. More daunting, perhaps, from a research perspective is that such effects are probably gene-specific. Nevertheless, in hepatoma cells, PKA has been shown to stabilize the interaction of the ligand-bound GR with DNA, which may require the involvement of the transcription factor, hepatocyte nuclear factor 3, whose binding to DNA is similarly stabilized (Espinas *et al*., 1995).

19.2.9 The epithelium as a site of action for LABAs

Although the non-bronchodilator actions of β_2-adrenoceptor agonists are of modest clinical relevance in the management of asthma and COPD (cf. airway smooth muscle relaxation), their ability to augment the efficacy of ICSs to a level that cannot be achieved by the corticosteroid alone is a highly important and extremely relevant clinical observation. The balance of evidence now indicates that LABAs, at least in part, enhance the anti-inflammatory effect of ICSs. In this respect LABAs, which are not anti-inflammatory *per se*, may be likened to powerful positive 'allosteric' modulators, which modify the conformation of ligand-bound GR (and/or the binding of necessary co-factors and co-activators) such that it interacts with DNA in a manner that is optimized for the transcription of anti-inflammatory genes. Moreover, it is likely that the airway epithelium is a major site of such 'allosterism'.

19.3 The muscarinic receptor as a therapeutic target

19.3.1 ACh as a pro-inflammatory mediator in COPD

N-Quaternary muscarinic receptor antagonists (or anticholinergics) such as ipratropium bromide (*Atrovent*®), oxitropium bromide (*Oxivent*®) and the long-acting compound, tiotropium bromide (*Spivira*®), are recommended as drugs of choice in many treatment guidelines for COPD as they effect rapid bronchodilatation, improve dyspnoea and enhance quality of life. Indeed, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) recommends muscarinic receptor antagonists for all stages of COPD. In clinical studies, these drugs show at least equivalent efficacy as bronchodilators when compared to conventional

β_2-adrenoceptor agonists (Gross, 1988; Lefcoe *et al*., 1982; Tashkin *et al*., 1986). Moreover, the efficacy of these drugs can relate to the degree of vagal tone in the airway, which may be significantly increased in patients with COPD (Gross *et al*., 1989). In addition to providing symptomatic relief, muscarinic receptor antagonists may evoke therapeutic effects, demonstrable experimentally and in clinical practice, that are not simply explained by bronchodilatation. In particular, these drugs reduce the number of hospitalizations due to exacerbations, and the duration of a hospitalization (Barr *et al*., 2006; Brusasco *et al*., 2003; Casaburi *et al*., 2002; Niewoehner *et al*., 2005; Powrie *et al*., 2007; Vincken *et al*., 2002). Ipratropium has been reported to further reduce exacerbations in patients with COPD taking salmeterol or salbutamol (Friedman *et al*., 1999; van Noord *et al*., 2000). With tiotropium, a reduction in the decline in lung function has also been suggested but not established (Casaburi *et al*., 2002). These chronic indices of efficacy are conventionally associated with an anti-inflammatory effect of a drug rather than simple bronchodilatation (Disse, 2001), suggesting that the parasympathetic nervous system can modulate pro-inflammatory cell function in COPD. Of major significance is the knowledge that ACh is synthesized by non-neuronal cells including airway epithelial cells (Koyama *et al*., 1992, 1998; Proskocil *et al*., 2004), T-lymphocytes, B-lymphocytes, mast cells, monocytes/macrophages, neutrophils and eosinophils (see Gosens *et al*., 2006; Wessler and Kirkpatrick, 2001). Moreover, a 14-fold increase in tissue ACh content has been reported in chronic inflammatory skin diseases, including atopic dermatitis (Wessler *et al*., 2003). Thus, an association between enhanced tissue ACh content and mucosal inflammation is not unprecedented. Based on these data it is possible that certain pro-inflammatory and immune cells are activated in COPD by a receptor-mediated process in response to an exaggerated release of ACh from neuronal and non-neuronal sources. Furthermore, nicotine from cigarette smoke could amplify the pro-inflammatory effect of endogenous ACh by acting at nicotinic receptors (typically, but not exclusively, α_7) on a number of pro-inflammatory and immune cells (de Jonge and Ulloa, 2007) including the airway epithelium (Klapproth *et al*., 1998; Plummer *et al*., 2005; Proskocil *et al*., 2004).

19.3.2 ACh and the airway epithelium

Bronchial airway epithelial cells are efficient producers of, and reservoirs for, non-neuronal ACh Indeed, in the presence of neostigmine, which blocks the degradation of ACh by cholinesterases, monkey bronchial epithelial cells are reported to secrete, spontaneously, ACh (Proskocil *et al*., 2004). Choline acetyltransferase (ChAT), which catalyses the transfer of an acetyl group from acetyl-co-enzyme A to choline to form ACh, also has been convincingly demonstrated in ciliary, secretory and brush border epithelium as well as in epithelial cells of submucosal glands (see Wessler and Kirkpatrick 2001; Figure 19.4). However, unlike neurons, airway epithelial cells do not have the capacity to concentrate ACh as they are devoid of cholinergic vesicles. Accordingly, the epithelial cell ACh content is considerably lower than that found in cholinergic nerve terminals. Epithelial cells also express a high-affinity choline transporter, several nicotinic receptor subunits (α_4, α_7, β_2 and α_3, α_5, β_2, β_4 in monkey and human respectively) and the muscarinic M_1- and M_3-receptor subtypes (Gosens *et al*., 2006; Maus *et al*., 1998; Proskocil *et al*., 2004).

Taken together the available data suggest that airway epithelial cells can both secrete ACh and be activated in an autocrine and/or paracrine manner by ACh released from other neuronal

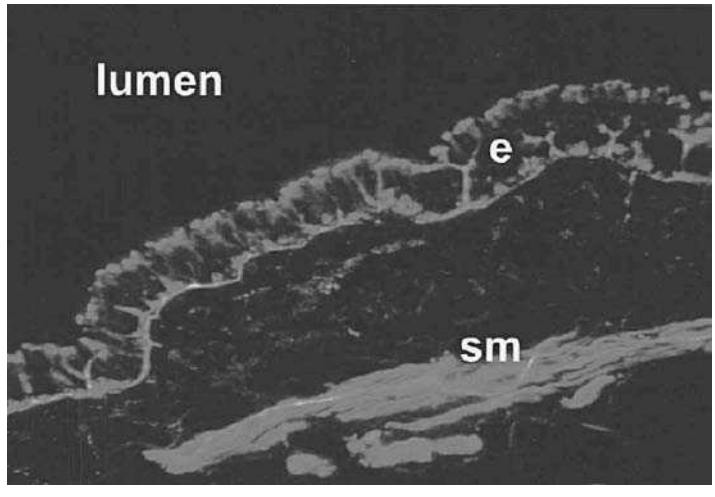


Figure 19.4 Human bronchial epithelium (e) and underlying smooth muscle (SM) stained with a rabbit polyclonal anti-ChAT antibody. Magnification ×200. Reproduced from Pulmonary Pharmacology and Therapeutics, 14, Fig 2, pg. 427, © Elsevier, 2001, with permission from Elsevier

and non-neuronal sources. Indeed, exposure of airway epithelial cells to ACh results in the release of a variety of pro-inflammatory mediators including GM-CSF (Klapproth *et al*., 1998), 15*S*-hydroxy-5Z,8Z,11Z,13E-eicosatetraeoic acid (Salari and Chan-Yeung, 1989) and neutrophil, eosinophil and monocyte chemotactic factors, such as leukotriene B_4 (Koyama *et al*., 1992, 1998). It is tempting to speculate that anticholinergic drugs, especially those with a long duration of action such as tiotropium, may antagonize the muscarinic actions of ACh on the airway epithelium and other target tissues and so attenuate inflammation. Such an effect could theoretically account for those beneficial effects (i.e. reduced hospitalization times) that cannot easily be explained by bronchodilatation.

19.4 Concluding remarks

It is now readily appreciated that the airway epithelium plays a complex role in health and disease. Clearly, the epithelial cell layer provides a physical protective barrier between the airway lumen and the underlying smooth muscle (both bronchial and vascular); it also has impressive metabolic functions where it can degrade bio-active compounds such as histamine and neurokinins. In contrast, the airway epithelium is intimately involved in the pathogenesis of chronic inflammation that characterizes asthma and COPD, and, thus, provides a primary target for the therapeutic actions of ICSs. What now is clearly apparent is the importance of the airway epithelium as a site of action for other drugs used routinely in the treatment of respiratory diseases. This is certainly true for LABAs in their capacity to enhance the anti-inflammatory actions of ICSs, but could also apply to long-acting anticholinergics, which may have a negative impact on airway inflammation by blocking the muscarinic actions of neuronal and non-neuronal ACh. Further research in this area clearly is required to formally identify the receptors (muscarinic and/or nicotinic) responsible for mediating potentially

adverse effects of ACh as this could allow, in the future, the development of a novel class of bronchodilator with improved anti-inflammatory efficacy.

Acknowledgements

The author is an Alberta Heritage Foundation for Medical Research (AHFMR) Senior Scholar and is funded by the Canadian Institutes of Health Research.

References

Abraham G, Kneuer C, Ehrhardt C, Honscha W and Ungemach FR (2004) Expression of functional β_2-adrenergic receptors in the lung epithelial cell lines 16HBE14o-, Calu-3 and A549. *Biochimica et Biophysica Acta*, **1691**, 169–179.

Barnes NC, Qiu YS, Pavord ID, Parker D, Davis PA, Zhu J, Johnson M, Thomson NC and Jeffery PK (2006) Anti-inflammatory effects of salmeterol/fluticasone propionate in chronic obstructive lung disease. *American Journal of Respiratory and Critical Care Medicine*, **173**, 736–743.

Barnes PJ (1996) Pathophysiology of asthma. *British Journal of Clinical Pharmacology*, **42**, 3–10.

Barnes PJ (2001a) Clinical outcome of adding long-acting β-agonists to inhaled corticosteroids. *Respiratory Medicine*, **95** (Suppl B), S12–S16.

Barnes PJ (2001b) Molecular mechanisms of corticosteroids in allergic diseases. *Allergy*, **56**, 928–936.

Barr RG, Bourbeau J, Camargo CA and Ram FS (2006) Tiotropium for stable chronic obstructive pulmonary disease: a meta-analysis. *Thorax*, **61**, 854–862.

Brusasco V, Hodder R, Miravitlles M, Korducki L, Towse L and Kesten S (2003) Health outcomes following treatment for six months with once daily tiotropium compared with twice daily salmeterol in patients with COPD. *Thorax*, **58**, 399–404.

Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, Yates JC and Vestbo J (2007) Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *New England Journal of Medicine*, **356**, 775–789.

Casaburi R, Mahler DA, Jones PW, Wanner A, San PG, ZuWallack RL, Menjoge SS, Serby CW and Witek T (2002) A long-term evaluation of once-daily inhaled tiotropium in chronic obstructive pulmonary disease. *European Respiratory Journal*, **19**, 217–224.

Cenni B and Picard D (1999) Ligand-independent activation of steroid receptors: new roles for old players. *Trends in Endocrinology and Metabolism*, **10**, 41–46.

Chivers JE, Cambridge LM, Catley MC, Mak JC, Donnelly LE, Barnes PJ and Newton R (2004) Differential effects of RU486 reveal distinct mechanisms for glucocorticoid repression of prostaglandin E release. *European Journal of Biochemistry*, **271**, 4042–4052.

Chong LK, Drury DE, Dummer JF, Ghahramani P, Schleimer RP and Peachell PT (1997) Protection by dexamethasone of the functional desensitization to β_2-adrenoceptor-mediated responses in human lung mast cells. *British Journal of Pharmacology*, **121**, 717–722.

Clark AR (2003) MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? *Journal of Endocrinology*, **178**, 5–12.

de Jonge WJ and Ulloa L (2007) The α_7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. *British Journal of Pharmacology*, **151**, 915.

Devalia JL, Sapsford RJ, Rusznak C, Toumbis MJ and Davies RJ (1992) The effects of salmeterol and salbutamol on ciliary beat frequency of cultured human bronchial epithelial cells, *in vitro*. *Pulmonary Pharmacology*, **5**, 257–263.

Disse B (2001) Anti-muscarinic treatment for lung diseases from research to clinical practice. *Life Sciences*, **68**, 2557–2564.

Dong Y, Aronsson M, Gustafsson JA and Okret S (1989) The mechanism of cAMP-induced glucocorticoid receptor expression. Correlation to cellular glucocorticoid response. *Journal of Biological Chemistry*, **264**, 13679–13683.

Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC and Zuraw BL (2007) The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *Journal of Allergy and Clinical Immunology*, **119**, 115–122.

Edwards MR, Johnson MW and Johnston SL (2006) Combination therapy: synergistic suppression of virus-induced chemokines in airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, **34**, 616–624.

Eickelberg O, Roth M, Lorx R, Bruce V, Rudiger J, Johnson M and Block LH (1999) Ligand-independent activation of the glucocorticoid receptor by β_2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *Journal of Biological Chemistry*, **274**, 1005–1010.

Espinas ML, Roux J, Pictet R and Grange T (1995) Glucocorticoids and protein kinase A co-ordinately modulate transcription factor recruitment at a glucocorticoid-responsive unit. *Molecular and Cellular Biology*, **15**, 5346–5354.

Foster WM, Bergofsky EH, Bohning DE, Lippmann M and Albert RE (1976) Effect of adrenergic agents and their mode of action on mucociliary clearance in man. *Journal of Applied Physiology*, **41**, 146–152.

Friedman M, Serby CW, Menjoge SS, Wilson JD, Hilleman DE and Witek TJ (1999) Pharmacoeconomic evaluation of a combination of ipratropium plus albuterol compared with ipratropium alone and albuterol alone in COPD. *Chest*, **115**, 635–641.

Giembycz MA and Newton R (2006) Beyond the dogma: novel β_2-adrenoceptor signalling in the airways. *European Respiratory Journal*, **27**, 1286–1306.

Gizycki MJ, Hattotuwa KL, Barnes N and Jeffery PK (2002) Effects of fluticasone propionate on inflammatory cells in COPD: an ultrastructural examination of endobronchial biopsy tissue. *Thorax*, **57**, 799–803.

Gormand F, Cheria-Sammari S, Aloui R, Guibert B, Malicier D, Perrin-Fayolle M, Lagarde M and Pacheco Y (1995) Granulocyte-macrophage colony stimulating factors (GM-CSF) and interleukin 8 (IL-8) production by human bronchial epithelial cells (HBEC) in asthmatics and controls. Lack of *in vitro* effect of salbutamol compared to sodium nedocromil. *Pulmonary Pharmacology*, **8**, 107–113.

Gosens R, Zaagsma J, Meurs H and Halayko AJ (2006) Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respiratory Research*, **7**, 73–88.

Greening AP, Ind PW, Northfield M and Shaw G (1994) Added salmeterol versus higher-dose corticosteroid in asthma patients with symptoms on existing inhaled corticosteroid. Allen & Hanburys Limited UK Study Group. *The Lancet*, **344**, 219–224.

Gross NJ (1988) Ipratropium bromide. *New England Journal of Medicine*, **319**, 486–494.

Gross NJ, Co E and Skorodin MS (1989) Cholinergic bronchomotor tone in COPD. Estimates of its amount in comparison with that in normal subjects. *Chest*, **96**, 984–987.

Hastie AT, Everts KB, Shaver JR, Cirelli R, Zangrilli J, Pollice MB, Fish JE and Peters SP (1997) β_2-agonist-elevated stress response in human bronchial epithelial cells *in vivo* and *in vitro*. *Lung*, **175**, 287–298.

Hattotuwa KL, Gizycki MJ, Ansari TW, Jeffery PK and Barnes NC (2002) The effects of inhaled fluticasone on airway inflammation in chronic obstructive pulmonary disease: a double-blind, placebo-controlled biopsy study. *American Journal of Respiratory and Critical Care Medicine*, **165**, 1592–1596.

Hirst MA, Northrop JP, Danielsen M and Ringold GM (1990) High level expression of wild type and variant mouse glucocorticoid receptors in Chinese hamster ovary cells. *Molecular Endocrinology*, **4**, 162–170.

Howarth PH, Beckett P and Dahl R (2000) The effect of long-acting β_2-agonists on airway inflammation in asthmatic patients. *Respiratory Medicine*, **94** (Suppl F), S22–S25.

Ind PW, Dal Negro R, Colman NC, Fletcher CP, Browning D and James MH (2003) Addition of salmeterol to fluticasone propionate treatment in moderate-to-severe asthma. *Respiratory Medicine*, **97**, 555–562.

Johnson M (2002) Effects of β_2-agonists on resident and infiltrating inflammatory cells. *Journal of Allergy and Clinical Immunology*, **110**, S282–S290.

Kalavantavanich K and Schramm CM (2000) Dexamethasone potentiates high-affinity β-agonist binding and Gsα protein expression in airway smooth muscle. *American Journal of Physiology – Lung Cell and Molecular Physiology*, **278**, L1101–L1106.

Kankaanranta H, Lahdensuo A, Moilanen E and Barnes PJ (2004) Add-on therapy options in asthma not adequately controlled by inhaled corticosteroids: a comprehensive review. *Respiratory Research*, **5**, 17–42.

Kaur M, Chivers JE, Giembyez MA and Newton R (2008) Long-acting β_2-adrenoceptor agonists Synergistically enhance glucocorticoid-dependent transcription in human airways epithelial and smooth muscle cell. *Mol. Pharmacol*., **73**, 203–214.

Keatings VM, Collins PD, Scott DM and Barnes PJ (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *American Journal of Respiratory and Critical Care Medicine*, **153**, 530–534.

Kelsen SG, Higgins NC, Zhou S, Mardini IA and Benovic JL (1995) Expression and function of the β-adrenergic receptor coupled-adenylyl cyclase system on human airway epithelial cells. *American Journal of Respiratory and Critical Care Medicine*, **152**, 1774–1783.

Kelsen SG, Anakwe O, Aksoy MO, Reddy PJ and Dhanasekaran N (1997a) IL-1β alters β-adrenergic receptor adenylyl cyclase system function in human airway epithelial cells. *American Journal of Physiology*, **273**, L694–L700.

Kelsen SG, Anakwe OO, Aksoy MO, Reddy PJ, Dhanesekaran N, Penn R and Benovic JL (1997b) Chronic effects of catecholamines on the β_2-adrenoreceptor system in cultured human airway epithelial cells. *American Journal of Physiology*, **272**, L916–L924.

Klapproth H, Racke K and Wessler I (1998) Acetylcholine and nicotine stimulate the release of granulocyte-macrophage colony stimulating factor from cultured human bronchial epithelial cells. *Naunyn Schmiedeberg's Archives of Pharmacology*, **357**, 472–475.

Korn SH, Wouters EF, Wesseling G, Arends JW and Thunnissen FB (1998) Interaction between glucocorticoids and β_2-agonists: α and β glucocorticoid-receptor mRNA expression in human bronchial epithelial cells. *Biochemical Pharmacology*, **56**, 1561–1569.

Korn SH, Jerre A and Brattsand R (2001) Effects of formoterol and budesonide on GM-CSF and IL-8 secretion by triggered human bronchial epithelial cells. *European Respiratory Journal*, **17**, 1070–1077.

Koyama S, Rennard SI and Robbins RA (1992) Acetylcholine stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. *American Journal of Physiology*, **262**, L466–L471.

Koyama S, Sato E, Nomura H, Kubo K, Nagai S and Izumi T (1998) Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity. *Journal of Applied Physiology*, **84**, 1528–1534.

Koyama S, Sato E, Masubuchi T, Takamizawa A, Kubo K, Nagai S and Isumi T (1999) Procaterol inhibits IL-1β- and TNFα-mediated epithelial cell eosinophil chemotactic activity. *European Respiratory Journal*, **14**, 767–775.

Lansley AB, Sanderson MJ and Dirksen ER (1992) Control of the beat cycle of respiratory tract cilia by Ca^{2+} and cAMP. *American Journal of Physiology*, **263**, L232–L242.

Lasa M, Abraham SM, Boucheron C, Saklatvala J and Clark AR (2002) Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Molecular and Cellular Biology*, **22**, 7802–7811.

Lee MH, Reynisdottir I and Massague J (1995) Cloning of $p57^{Kip2}$, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes and Development*, **9**, 639–649.

Lefcoe NM, Toogood JH, Blennerhassett G, Baskerville J and Paterson NA (1982) The addition of an aerosol anticholinergic to an oral β-agonist plus theophylline in asthma and bronchitis. A double-blind single dose study. *Chest*, **82**, 300–305.

Mak JC, Nishikawa M and Barnes PJ (1995a) Glucocorticosteroids increase β_2-adrenergic receptor transcription in human lung. *American Journal of Physiology*, **268**, L41–L46.

Mak JC, Nishikawa M, Shirasaki H, Miyayasu K and Barnes PJ (1995b) Protective effects of a glucocorticoid on down-regulation of pulmonary β_2-adrenergic receptors *in vivo*. *Journal of Clinical Investigation*, **96**, 99–106.

Maus AD, Pereira EF, Karachunski PI, Horton RM, Navaneetham D, Macklin K, Cortes WS, Albuquerque EX and Conti-Fine BM (1998) Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. *Molecular Pharmacology*, **54**, 779–788.

McGraw DW, Forbes SL, Mak JC, Witte DP, Carrigan PE, Leikauf GD and Liggett SB (2000) Transgenic over-expression of β_2-adrenergic receptors in airway epithelial cells decreases bronchoconstriction. *American Journal of Physiology – Lung Cell and Molecular Physiology*, **279**, L379–L389.

Miller-Larsson A and Selroos O (2006) Advances in asthma and COPD treatment: combination therapy with inhaled corticosteroids and long-acting β_2-agonists. *Current Pharmaceutical Design*, **12**, 3261–3279.

Mizzen LA and Welch WJ (1988) Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *Journal of Cell Biology*, **106**, 1105–1116.

Newton R (2000) Molecular mechanisms of glucocorticoid action: what is important? *Thorax*, **55**, 603–613.

Newton R, Seybold J, Kuitert LM, Bergmann M and Barnes PJ (1998) Repression of cyclooxygenase-2 and prostaglandin E_2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated mRNA. *Journal of Biological Chemistry*, **273**, 32312–32321.

Niewoehner DE, Rice K, Cote C, Paulson D, Cooper JA, Korducki L, Cassino C and Kesten S (2005) Prevention of exacerbations of chronic obstructive pulmonary disease with tiotropium, a once-daily inhaled anticholinergic bronchodilator: a randomized trial. *Annals of Internal Medicine*, **143**, 317–326.

Nishimura K, Tamaoki J, Isono K, Aoshiba K and Nagai A (2002) β-Adrenergic receptor-mediated growth of human airway epithelial cell lines. *European Respiratory Journal*, **20**, 353–358.

O'Byrne PM, Barnes PJ, Rodriguez-Roisin R, Runnerstrom E, Sandstrom T, Svensson K and Tattersfield A (2001) Low dose inhaled budesonide and formoterol in mild persistent asthma: the OPTIMA randomized trial. *American Journal of Respiratory and Critical Care Medicine*, **164**, 1392–1397.

O'Byrne PM, Bisgaard H, Godard PP, Pistolesi M, Palmqvist M, Zhu Y, Ekstrom T and Bateman ED (2005) Budesonide/formoterol combination therapy as both maintenance and reliever medication in asthma. *American Journal of Respiratory and Critical Care Medicine*, **171**, 129–136.

Oddera S, Silvestri M, Lantero S, Sacco O and Rossi GA (1998) Downregulation of the expression of intercellular adhesion molecule (ICAM)-1 on bronchial epithelial cells by fenoterol, a β_2-adrenoceptor agonist. *Journal of Asthma*, **35**, 401–408.

Oikarinen J, Hamalainen L and Oikarinen A (1984) Modulation of glucocorticoid receptor activity by cyclic nucleotides and its implications on the regulation of human skin fibroblast growth and protein synthesis. *Biochimica et Biophysica Acta*, **799**, 158–165.

Pang L and Knox AJ (2000) Synergistic inhibition by β_2-agonists and corticosteroids on tumor necrosis factor-α-induced interleukin-8 release from cultured human airway smooth-muscle cells. *American Journal of Respiratory Cell and Molecular Biology*, **23**, 79–85.

Pang L and Knox AJ (2001) Regulation of TNFα-induced eotaxin release from cultured human airway smooth muscle cells by β_2-agonists and corticosteroids. *FASEB Journal*, **15**, 261–269.

Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ and Ullman A (1997) Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and

Corticosteroids Establishing Therapy (FACET) International Study Group. *New England Journal of Medicine*, **337**, 1405–1411.

Penn RB, Kelsen SG and Benovic JL (1994) Regulation of β-agonist- and prostaglandin E_2-mediated adenylyl cyclase activity in human airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, **11**, 496–505.

Plummer HK, Dhar M and Schuller HM (2005) Expression of the α_7 nicotinic acetylcholine receptor in human lung cells. *Respiratory Research*, **6**, 29–37.

Powrie DJ, Wilkinson TM, Donaldson GC, Jones P, Scrine K, Viel K, Kesten S and Wedzicha JA (2007) Effect of tiotropium on sputum and serum inflammatory markers and exacerbations in chronic obstructive pulmonary disease. *European Respiratory Journal*, **30**, 472–478.

Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J and Spindel ER (2004) Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology*, **145**, 2498–2506.

Rangarajan PN, Umesono K and Evans RM (1992) Modulation of glucocorticoid receptor function by protein kinase A. *Molecular Endocrinology*, **6**, 1451–1457.

Roberts JA, Bradding P, Britten KM, Walls AF, Wilson S, Gratziou C, Holgate ST and Howarth PH (1999) The long-acting β_2-agonist salmeterol xinafoate: effects on airway inflammation in asthma. *European Respiratory Journal*, **14**, 275–282.

Roth M, Johnson PR, Rudiger JJ, King GG, Ge Q, Burgess JK, Anderson G, Tamm M and Black JL (2002) Interaction between glucocorticoids and β_2-agonists on bronchial airway smooth muscle cells through synchronised cellular signalling. *The Lancet*, **360**, 1293–1299.

Sabatini F, Silvestri M, Sale R, Serpero L, Di Blasi P and Rossi GA (2003) Cytokine release and adhesion molecule expression by stimulated human bronchial epithelial cells are down-regulated by salmeterol. *Respiratory Medicine*, **97**, 1052–1060.

Sackner MA, Epstein S and Wanner A (1976) Effect of β-adrenergic agonists aerosolized by freon propellant on tracheal mucous velocity and cardiac output. *Chest*, **69**, 593–598.

Salari H and Chan-Yeung M (1989) Release of 15-hydroxyeicosatetraenoic acid (15-HETE) and prostaglandin E_2 (PGE_2) by cultured human bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, **1**, 245–250.

Sanderson MJ and Dirksen ER (1989) Mechanosensitive and β-adrenergic control of the ciliary beat frequency of mammalian respiratory tract cells in culture. *American Review of Respiratory Disease*, **139**, 432–440.

Schwiebert LM, Stellato C and Schleimer RP (1996) The epithelium as a target of glucocorticoid action in the treatment of asthma. *American Journal of Respiratory and Critical Care Medicine*, **154**, S16–S19.

Sears MR and Taylor DR (1994) The β_2-agonist controversy. Observations, explanations and relationship to asthma epidemiology. *Drug Safety*, **11**, 259–283.

Shrewsbury S, Pyke S and Britton M (2000) Meta-analysis of increased dose of inhaled steroid or addition of salmeterol in symptomatic asthma (MIASMA). *British Medical Journal*, **320**, 1368–1373.

Singam R, Jena PK, Behera S, Hellermann GR, Lockey RF, Ledford D and Mohapatra SS (2006) Combined fluticasone propionate and salmeterol reduced RSV infection more effectively than either of them alone in allergen-sensitized mice. *Virology Journal*, **23**, 32–41.

Szapary D, Xu M and Simons SS (1996) Induction properties of a transiently transfected glucocorticoid-responsive gene vary with glucocorticoid receptor concentration. *Journal of Biological Chemistry*, **271**, 30576–30582.

Tashkin DP, Ashutosh K, Bleecker ER, Britt EJ, Cugell DW, Cummiskey JM, DeLorenzo L, Gilman MJ, Gross GN, Gross NJ, Kotch A, Lakshminarayan S, Maguire G, Miller M, Plummer A, Renzetti A, Sackner MA, Skorodin MS, Wanner A, Watanabe S (1986) Comparison of the anticholinergic bronchodilator ipratropium bromide with metaproterenol in chronic obstructive pulmonary disease. A 90-day multi-center study. *American Journal of Medicine*, **81**, 81–90.

Tse R, Marroquin BA, Dorscheid DR and White SR (2003) β-Adrenergic agonists inhibit corticosteroid-induced apoptosis of airway epithelial cells. *American Journal of Physiology – Lung Cell and Molecular Physiology*, **285**, L393–L404.

Usmani OS, Ito K, Maneechotesuwan K, Ito M, Johnson M, Barnes PJ and Adcock IM (2005) Glucocorticoid receptor nuclear translocation in airway cells after inhaled combination therapy. *American Journal of Respiratory and Critical Care Medicine*, **172**, 704–712.

van Noord JA, de Munck DR, Bantje TA, Hop WC, Akveld ML and Bommer AM (2000) Long-term treatment of chronic obstructive pulmonary disease with salmeterol and the additive effect of ipratropium. *European Respiratory Journal*, **15**, 878–885.

Verdugo P, Johnson NT and Tam PY (1980) β-Adrenergic stimulation of respiratory ciliary activity. *Journal of Applied Physiology*, **48**, 868–871.

Vincken W, van Noord JA, Greefhorst AP, Bantje TA, Kesten S, Korducki L and Cornelissen PJ (2002) Improved health outcomes in patients with COPD during 1 yr's treatment with tiotropium. *European Respiratory Journal*, **19**, 209–216.

Volonaki E, Psarras S, Xepapadaki P, Psomali D, Gourgiotis D and Papadopoulos NG (2006). Synergistic effects of fluticasone propionate and salmeterol on inhibiting rhinovirus-induced epithelial production of remodelling-associated growth factors. *Clinical and Experimental Allergy*, **36**, 1268–1273.

Weigel NL and Zhang Y (1998) Ligand-independent activation of steroid hormone receptors. *Journal of Molecular Medicine*, **76**, 469–479.

Wessler IK and Kirkpatrick CJ (2001) The non-neuronal cholinergic system: an emerging drug target in the airways. *Pulmonary Pharmacology and Therapeutics*, **14**, 423–434.

Wessler I, Reinheimer T, Kilbinger H, Bittinger F, Kirkpatrick CJ, Saloga J and Knop J (2003) Increased acetylcholine levels in skin biopsies of patients with atopic dermatitis. *Life Sciences*, **72**, 2169–2172.

Woolcock A, Lundback B, Ringdal N and Jacques LA (1996) Comparison of addition of salmeterol to inhaled steroids with doubling of the dose of inhaled steroids. *American Journal of Respiratory and Critical Care Medicine*, **153**, 1481–1488.

Yang B, Schlosser RJ and McCaffrey TV (1996) Dual signal transduction mechanisms modulate ciliary beat frequency in upper airway epithelium. *American Journal of Physiology*, **270**, L745–L751.

Yeates DB, Spektor DM and Pitt BR (1986) Effect of orally administered orciprenaline on tracheobronchial mucociliary clearance. *European Journal of Respiratory Disease*, **69**, 100–108.

Zhang S, Jonklaas J and Danielsen M (2007) The glucocorticoid agonist activities of mifepristone (RU486) and progesterone are dependent on glucocorticoid receptor levels but not on EC_{50} values. *Steroids*, **72**, 600–608.

20

Pulmonary Delivery of Novel Therapies

Bruce Rubin[1] and **James B. Fink**[2]
[1] *Departments of Pediatrics and Biomedical Engineering, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA,*
[2] *Fellow, Respiratory Science, Nektar Therapeutics, San Carlos, CA, USA*

20.1 Introduction

The lung has long been a target for drug delivery. Aerosol delivery to the pulmonary airway has been shown to be as effective as a 50–100-fold greater dose of the same medication, delivered systemically (Newhouse and Corkery, 2001). This high therapeutic index provides local therapeutic benefit while reducing associated side effects.

Historically, delivery via inhalation has been targeted to the conducting airway. The lung is now also thought to be a good site for delivery of proteins and peptides for both local and systemic therapy. The large surface area of the lung parenchyma ($100\,m^2$) provides a highly permeable barrier (0.2–0.7 μm) between air and blood compared to the surface area of the conducting airway ($3\,m^2$) or the skin (less than $2\,m^2$) (Patton, 1996). While the biology of the lung makes it an attractive target for noninvasive drug delivery, many mechanisms function to prevent airborne particles from entering the body. Medical aerosols must circumvent or bypass airway defences to reach their intended targets within the airway and lungs. Once inhaled, drugs deposited in the airway either exert local actions, are absorbed through the airway passing into the circulation for system action, are cleared from the lungs, or are degraded via drug metabolism.

Advances in our knowledge of transport systems, and improved aerosol delivery systems, holds the promise of improved drug bioavailability. In order for inhalation to offer a viable alternative to parenteral routes for protein administration, we need to be able to control the distribution, absorption, metabolism and clearance of drugs from the airway.

Inhalation technology can improve the ability to deliver proteins and peptides. Preserving the molecular integrity of a formulation and delivering it to the appropriate target in the lung

The Pulmonary Epithelium in Health and Disease Edited by David Proud
 ISBN: 978-0-470-05951-7

are critical for effective therapy. Aerosol characteristics and breathing patterns can help to target an aerosol to specific regions of the lung, from central airway to the acinus.

20.2 Drug targeting

Particle size affects deposition patterns, clearance, safety and physicochemical properties. Particles with mass mean aerodynamic (MMAD) greater than 5 μm tend to deposit in the upper airway, and are considered nonrespirable, while those 0.5 to 5 μm are considered the respirable fraction with distribution in the central and peripheral airway. Ultrafine particles, less than 100 nm, readily translocate across the pulmonary epithelium into the bloodstream and appear to bypass macrophage clearance (Kreyling *et al*., 2006) but have been associated with toxicity (Oberdoster *et al*., 2005). Large porous particles with geometric diameters between 5 and 30 μm (and density less than water) have been shown to have pulmonary delivery efficiencies up to 60 per cent. These larger particles also appear to resist macrophage clearance (Dunbar *et al*., 2002).

The aerosol mode of administration can target medications to airway secretions, the epithelial cells, or systemically. There are more than 70 aerosol products for therapeutic inhalation, consisting of more than 20 active ingredients. Today, most medications are targeted to the airway epithelium, including the neuromuscular plexus (bronchodilators) and inflammatory cells (corticosteroids). Drugs such as the $P2Y_2$ ion channel activators are targeted directly to the ciliated epithelium. Mucolytics, proteases, and antibiotics are targeted to secretions in the airway rather than to the epithelial cells.

In order for a drug to exert its desired effect, it needs to reach the target surface. This could be the mucus layer, receptors on the pulmonary epithelial surface, or absorption through the cell and the endothelium. Upon reaching the lung periphery, barriers include surface lining fluid, lung surfactant, epithelium, interstitium, basement membrane and the pulmonary endothelium (Figure 20.1).

Very small particles targeted to the alveolus can be effective for systemic delivery of macromolecules through the extensive pulmonary vascular bed. Insulin was the first

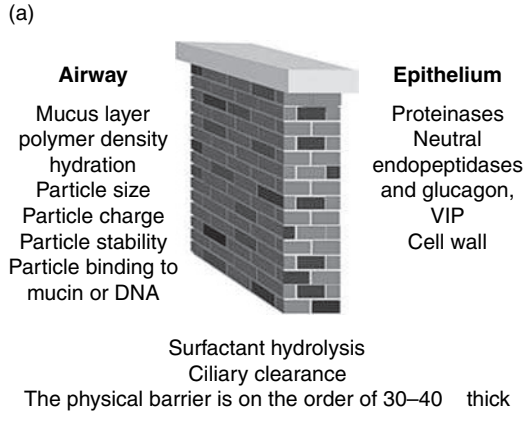


Figure 20.1 (a) Barriers to epithelial drug delivery to the lung. (b) Proposed means of overcoming these barriers

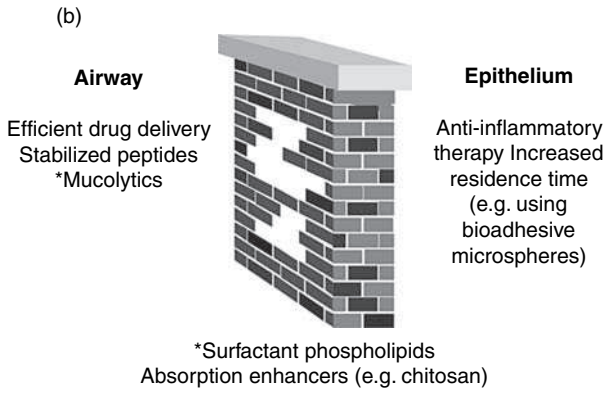


Figure 20.1 (Continued)

medication introduced for systemic administration through aerosol administration, but other peptides and macromolecules are under development. Considerations for systemic administration include cost, convenience, efficacy, and safety (Mallet *et al*., 1997).

20.2.1 Delivery to the conducting airway

Topical administration of inhaled aerosols have been commonly used to treat airway diseases (Table 20.1). In the central airway the role of the bronchial circulation in redistribution of inhaled drugs and the absorption and clearance of a specific inhaled drug can influence efficacy and safety. The presence of specific receptors in the airway enable targeting aerosol delivery with drugs that bind to these receptors. Receptors for β_2-agonists (such as albuterol) are present in high density in the airway epithelium from large bronchi through the terminal bronchioles (Usmani *et al*., 2005). Airway smooth muscle has a greater density of β-receptors

Table 20.1 A list of medications now approved for aerosol delivery (*), and under investigation for the treatment of pulmonary diseases

*Antibacterials
*Antivirals
Antifungals
*Bronchodilators
Immunosuppressive drugs
*Non-steroidal anti-inflammatory drugs
*Steroids
Surfactants
siRNAs
*Prostaglandins
*Mucolytics
Mucokinetics
Antitussives
Expectorants
Gene therapy vectors

in the bronchioles than the bronchi (Carstairs *et al*., 1985) The alveolar wall contains >90% of all β-receptors and in this location they are thought to regulate fluid absorption (Berthiaume *et al*., 1987). For use as a bronchodilator, therefore, β_2-agonists should be targeted to the conducting airway.

Muscarinic M3 and M1 receptors (target of anticholinergic medications such as ipratropium and tiotropium bromide) are found in high density in submucosal glands and airway ganglia, with lower density in smooth muscles throughout the airway, nerves in intrapulmonary bronchi and in alveolar walls (Mak and Barnes, 1990) This suggests that for greatest efficacy, anticholinergic medications should be targeted to the larger conducting airway.

A small amount of histamine aerosol deposited predominantly in the large conducting airway is as effective as an order of magnitude larger dose delivered diffusely, suggesting that histamine receptors are primarily in the conducting airway and surface concentration affects response (Ruffin *et al*., 1978).

Anti-inflammatory therapy is targeted to numerous effector cells, including eosinophils, macrophages, lymphocytes and dendritic cells throughout the airway and alveolar tissue (Carrol *et al*., 1997; Kraft *et al*., 1996). It is now recognized that both the airway epithelum (Chanez *et al*., 2004) and the airway smooth muscle (Panettari, 2004) can also secrete inflammatory mediators when stimulated and may play a much larger role in the progression of inflammatory airway diseases than once realized.

20.2.2 Systemic delivery

Systemic delivery of macromolecules has principally been limited to intravenous, intramuscular, and subcutaneous routes. Oral administration does not work well for many macromolecules, as proteins are often digested before they can be absorbed into the bloodstream. The large size of these molecules limit absorption through nasal mucosa and skin without the use of penetration enhancers (Illum and Fischer, 1997).

Systemic delivery of some macromolecule (protein) drugs via the pulmonary route can provide a higher bioavailability than other noninvasive ports of entry (10–200 times greater than nasal and gastrointestinal) (Table 20.2). This has been attributed to the enormous

Table 20.2 Medications currently being used (*) and under investigation for systemic delivery by pulmonary aerosol administration

*Insulin
Heparin
Levodopa
Ergotamine
Calcitonin
Human growth hormone (hGH)
Sildenofil
Antiproteases (alpha-1 antitrypsin)
*Vaccines
Gene therapy vectors
Morphine
Fentenyl
Alprazolam

surface area, thin diffusion layer, slow surface clearance, and slow antiprotease defence system (Patton, 1996). Since 1925, it has been known that proteins, such as insulin (with a molecular weight of 5.7 kDa), delivered as aerosol can be absorbed from the lung (Gaensslen, 1925). The larger the protein, however, the greater the barrier to absorption. Macromolecules greater than 40 kDa (5–6 nm in diameter) tend to be slowly absorbed over many hours; for example inhaled α_1-antitrypsin (45–51 kDa) has a T_{max} of up to 48 hours (Byron and Patton, 1994).

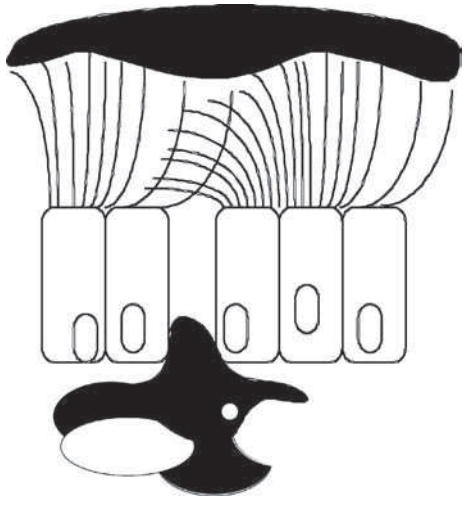
Inhaled proteins and peptides have a more rapid onset of action than occurs with subcutaneous administration, but have a relative bioavailability only 8–22 per cent of that for subcutaneous injection (Kim *et al*., 2003). This suggests that achieving equivalent systemic doses by inhalation requires more drug to be given, resulting in potentially greater acute effects, and also higher cost of goods when given by inhalation.

These limitations have stimulated efforts to enhance permeability and slow enzymatic degradation. Permeation enhancers are not required for inhalation, but may increase bioavailability. Early enhancers (e.g., surfactants, fatty acids, bile salts, citric acids) altered the curvature of the phospholipid-based pulmonary epithelium, but raised safety concerns for the integrity of epithelium (Okamoto *et al*., 2002). Tetradecyl-β-maltoside (TDM) is a nonionic detergent that can briefly open tight junctions. TDM has a low critical micelle concentration (CMC) relative to bile salts and other charged surfactants, with permeation activity observed at lower concentrations, as shown for low molecular weight heparin (Yang *et al*., 2004) and insulin (Hussain *et al*., 2006). High molecular weight polymers, such as chitosan, with bioadhesive properties also show promise for enhancing particle retention and absorption (Yamada *et al*., 2005).

20.3 Removal of aerosol particles from the lung

Airway surface fluid is composed of the pericilliary fluid lubricating the cilia below a more viscous mucous gel layer (Figure 20.2). For aerosol particles to settle on the conducting airway, they must pass through this layer to reach the epithelium. Absorption of aerosols may be limited by mucus clearance from the airway. Particles deposited in the conducting airway are largely removed through mucociliary clearance. The rate of mucus clearance varies with the region of the airway, the number of ciliated cells, their beat frequency and stroke power, as well as the quality and quantity of mucus. Ciliated epithelial cells cover 30–65 per cent of the airway epithelial surface (Blake and Sleigh, 1974). Mucus transport velocity decreases as the percentage of these cells decreases between the trachea ($\sim$ 50 per cent) to the fifth generation of the airway ($\sim$ 15 per cent) (Serafini and Michaelson, 1977). In lung disease, mucociliary clearance is reduced by impaired ciliary function, and/or quantity and consistency of mucus, decreasing clearance of aerosols deposited in the conducting airway.

Soluble particles can be absorbed in the conducting airway (Edsbacker, 2002). From the submucosal region, particles are absorbed into either the systemic circulation, the bronchial circulation or the lymphatic system. Lipophilic molecules pass easily through the airway epithelium via passive transport. Hydrophilic molecules cross via extracellular pathways, such as through tight junctions, or by active transport via endocytosis (Summers, 1991).



Mucous layer
This contains mucin, DNA and action polymers, lipids, peptides, and products of inflammation when present.
Therapies:
- Mucolytics
- Antiproteases, antioxidants, antielastases
- Anti-inflammatory agents
- Antibiotics

Periciliary fluid layer
Fluid between mucus and epithelium, Composition is thought to be isotonic. Surfactant separates periciliary fluid and mucus
Therapies:
- Anti-inflammatory agents
- Mucokinetic and surfactant

Epithelial cells
Principally consists of goblet cells and ciliated epithelial cells
Therapies:
- Antioxidants
- Genetransfer
- Genemodifiers
- Growth factors and regeneration factors
- Ion transport modifiers

Submucous glands
Consists of serous and mucous gland cells
Therapies:
- Mucoregulatory agents
- Genetransfer
- Genemodifiers

Figure 20.2 Medications can be targeted to different layers in the airway depending on their intended use and their ability to be transported into or through these layers. This diagram shows the primary component layers of the surface epithelium in the large conducting airway and medications that are now or potentially could be targeted to these layers

20.3.1 Alveolar macrophages

Aerosol particles deposited in the alveolar and terminal airway space can be subject to absorptive or non-absorptive removal. Absorptive removal includes uptake by macrophages and epithelial cells (Sibile and Reynolds, 1990). Alveolar macrophages are the predominant phagocytic cell in the distal airway for the lung defence against inhaled micro-organisms, particles and other toxic agents. There are up to seven alveolar macrophages per alveolus in the lungs of healthy nonsmokers (Stone *et al*., 1992). Macrophages ingest insoluble particles that are deposited in the alveolar region and are then either cleared by the lymphatic system, or moved into the ciliated airway along currents in alveolar fluid and then cleared via the mucociliary escalator (Folkesson *et al*., 1996). This process can take months to complete.

The adhesion of airborne particles to alveolar macrophages is mediated through electrostatic interaction, or via specific receptors. Particles are then internalized through surface cavitation, or vacuole and pseudopod formation (Stossel, 1977). In the case of proteins, internalization is followed by further digestion (metabolization) by peptidases. Activated macrophages may then secrete a variety of cytokines and chemokines and can migrate to the ciliated airway epithelium for transport via mucociliary clearance or penetrate through the respiratory epithelium into the interstitial space (Sorokin, 1970).

The internalization of airborne particles depends on the particle size, and on the composition of coating material. Both features can be used to selectively control drug uptake by alveolar macrophages. While particles of 3 μm diameter are better internalized than 6 μm particles, particles smaller than 0.26 μm appear to be much less actively ingested by macrophages (Lauweryns and Baert, 1977).

20.4 Barriers to drug absorption from the lung

20.4.1 Epithelial barrier

Systemic uptake can be enhanced by delivering the drug to the deep lung where it can translocate across the alveolar epithelium. Systemic delivery of some large molecules, usually proteins, via the pulmonary route provides a higher bioavailability than other noninvasive ports of entry. There are large quantitative differences in the transepithelial transport of compounds in the upper airway as compared to to the lower respiratory tract. The relative contributions of absorption across the large airway and translocation through alveoli are important to local and systemic delivery. Transport of drugs across the epithelium of the conducting airway is limited by a smaller surface area, and limited regional blood flow. With standard aerosol devices, the upper airway filter out 70–90 per cent of medical aerosols with the remainder reaching the parynchema (Groneberg *et al*., 2003).

The pulmonary epithelium is the limiting barrier for protein absorption. The mechanisms by which macromolecules are absorbed across the alveolar capillary membrane remain poorly understood. The normal air/blood barrier appears to differentially restrict the passage of large proteins, as evident by the finding that concentrations of macromolecules in bronchoalveolar lavage fluid (BALF) are extremely low compared with smaller proteins like albumin (Holter *et al*., 1986). Understanding how exogenous and endogenous proteins traverse the air–blood barriers of the lung is likely to improve the ability to deliver drugs from the lung into the systemic circulation and target drugs to lung parenchymal cells.

The pulmonary blood–gas barrier is composed of the alveolar epithelium, capillary endothelium, and the extracellular matrix consisting of basement membranes of the two cell layers. The distal air spaces of the lung are lined with a continuous epithelium comprising type I and II alveolar epithelial cells, joined by tight junctions, which help to keep the air spaces relatively dry for efficient gas exchange. Type I pneumocytes account for 95 per cent of the alveolar surface (Groneberg *et al*., 2003). Intercellular junctions differ between alveolar type 1 epithelium and alveolar capillary endothelial cells. Type I cells have smaller pore size (0.6–1.0 nm) and greater tight junction depth (0.26 μm) than endothelial cells (4–5.8 nm and 0.17 μm, respectively) and this has been associated with 1000 times lower epithelial permeability to substances such as sucrose (Taylor and Gaar, 1970). Endocytotic vesicles may function as carriers in the absorption process of larger proteins such as insulin. While the main recognized function of type II pneumocytes is the production of surfactant proteins and differentiation into type I cells, they also express a number of transport proteins and receptors (Taylor and Gaar, 1970).

Three other types of tight junction have been identified for extra- and intrapulmonary airway, differing in the degree of luminal fibril interconnections. Type I tight junctions are largely found between extrapulmonary ciliated cells. Type II junctions are primarily present

in smaller airway and between Clara cells. Type III tight junctions are found between mucous cells with a secretory cycle-associated change in permeability (more leaky during active state of secretion) (Inoue *et al*., 1976). It is likely that the regional differences in tight junction morphology are more directly linked to transepithelial transport capacities of water and ions than to larger molecules such as proteins.

It has been hypothesized that proteins, and other large molecules, pass through the epithelium by absorptive receptor mediated transcytosis, by paracellular transport between bijunctions or trijunctions, or through large pores in the epithelium caused by cell injury. Transcytosis facilitates passage of a macromolecule drug into the bloodstream with relatively high bioavailability. Although the function of transcytosis in normal lung homeostasis is uncertain, it is thought to be a natural mechanism for keeping the volume of alveolar fluid appropriate, and for removing endogenous proteins that leak into alveoli across the epithelium.

In the process of transcytosis, small invaginations of the epithelial membrane develop. These microscopic 'bubbles' expand into the alveolar surface of the cell until they separate from the cell membrane, carrying with them a small amount of alveolar fluid containing the dissolved protein. This transcytotic vesicle moves from the alveolar surface of the epithelial cell to the interstitial surface, where it releases its contents into the interstitium of the lung.

Transcytosis involving caveolae and clathrin-coated pits is likely the main route of alveolar epithelial protein transport. Caveolae are small plasma membrane invaginations with a cytoplasmically oriented protein coat (Rathberg *et al*., 1992). Three genes for caveolin have been cloned and are regulated and expressed in a tissue-specific manner (Ralston and Ploug, 1999). Multiple isoforms of caveolin are expressed in the same cell with distinct distributions implying functionally distinct subgroups that may be involved in transcytosis or the vesicular movement of macromolecules across endothelial cells (Schnitzer *et al*., 1995). Both Type I and Type II cells have membrane apical and basolateral invaginations consistent with vesicular or caveolar transport capabilities. Horseradish peroxidase, a pinocytosis marker, exhibits low and symmetric permeability across the alveolar epithelium (Matsukawa *et al*., 1996) suggesting that absorption of proteins/peptides via pinocytotic routes may be minimal. Ultrastructural protein tracers (e.g. horseradish peroxidase and cytochrome c) generally do not cross the epithelial junctions but are found in the interstitial spaces when perfused in the vasculature. Exceptions to this are catalase and ferritin, which do not penetrate endothelial junctions. From the interstitial space, macromolecules may again traverse the capillary endothelial cell by transcytosis to get into the blood or, more likely, they are absorbed into the circulation through the junctions between the endothelial cells of the capillaries or venules.

Figure 20.3 is a schematic diagram illustrating protein transport processes in the alveolus (Kim and Malik, 2002). Specific receptors for albumin (60 kDa albumin-binding glycoprotein or gp60), immunoglobulin G (FcRn), and polymeric immunoglobulin A (pIgR) are shown. Other protein receptors not shown include those for insulin and transferrin, both of which are also expressed in basolateral membranes of alveolar epithelial cells. The albumin-binding protein, gp60, was first identified in endothelial cells and later found to be expressed in the alveolar epithelial cells. Albumin is transported across alveolar epithelium via gp60- and caveolae- (shown as pink circles) -mediated transcytosis (illustrated by the open, red-outlined arrow showing direction of net absorption). IgG appears to be similarly absorbed via FcRn-mediated transcytosis across the alveolar epithelium, shown in blue and open, blue-outlined

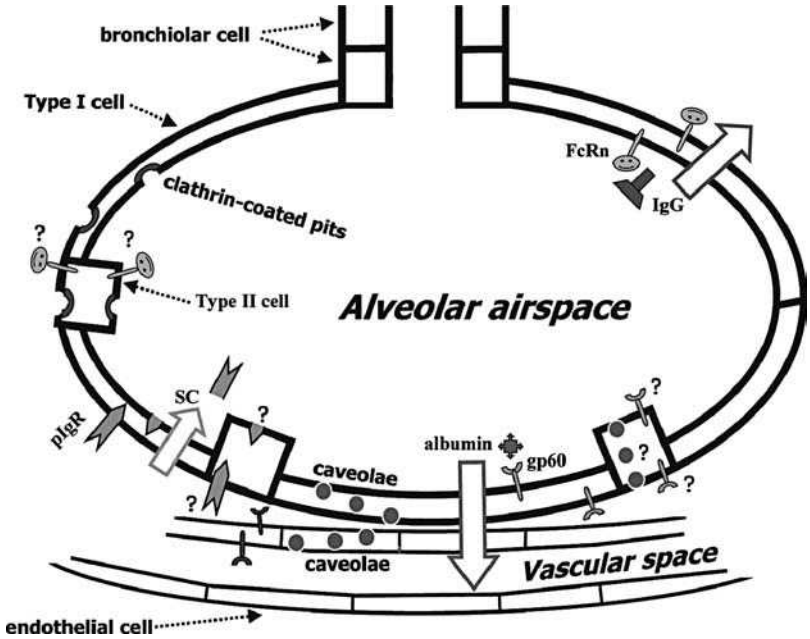


Figure 20.3 Diagram illustrating protein transport processes in the alveolar barrier. Specific receptors for albumin (gp60), immunoglobulin G (FcRn), and polymeric immunoglobulin A (pIgR) are indicated. Albumin is transported across the alveolar epithelium via gp60- and caveolae (shown as pink circles)-mediated transcytosis (illustrated by the open, red-outlined arrow showing direction of net absorption). IgG appears to be absorbed via FcRn-mediated transcytosis, shown in blue receptors and open, blue-outlined arrow for direction of absorption. Secretory component (SC; the extracellular portion of pIgR) is secreted into alveolar lining fluid (green receptors and direction of secretion). Question marks next to the receptors or processes denote that in type II cells these pathways have not been confirmed. From Kim, K.-J. *et al.*, *Am J Physiol Lung Cell Mol Physiol* 2003; **284**: L247–L259, reproduced with permission

arrow for direction of absorption. Secretory component (SC; the extracellular portion of pIgR) appears to be secreted into alveolar lining fluid (green receptors and direction of secretion).

Type I cells and endothelial cells are known to contain numerous vesicles and membrane invaginations (including caveolae and clathrin-coated pits) that are thought to play important roles in internalization of proteins and transcellular movement of cargo proteins. In the endothelium, caveolae structures outnumber clathrin-coated pit structures by 19 to 1. The relative contribution of transcytosis mediated by caveolae versus clathrin-coated pits to overall serum protein transport across alveolar epithelium is unknown. It is unclear whether type II cells have the ability to transport proteins via caveolae-mediated process involving gp60 and other albumin-binding proteins. It is important to note that restricted passive diffusion of large serum proteins (e.g., albumin, IgG, and pIgA) via the paracellular route plays an insignificant role in the net absorption or secretion of these proteins across normal alveolar epithelium. Under pathological conditions, inflammation and injury leads to paracellular leakage of those large proteins (Kim and Malik, 2002).

20.4.2 Protease and peptidase degradation

Lung protein clearance may also involve catabolic pathways. Degradation by peptidases localized at the apical surface of respiratory epithelial cells may be a mechanism for clearing peptides and some smaller proteins from lung air spaces. Alveolar protein clearance appears to be size-dependent; larger proteins clear at a slower rate. Proteins such as albumin are cleared relatively intact, while smaller proteins and peptides may undergo significant degradation (Kim and Malik, 2002). Peptides that have been chemically altered to resist protease activity exhibit higher pulmonary bioavailability suggesting involvement of proteases in peptide processing in the lung (Forges *et al*., 1999). Proteins with molecular weights between 6 and 50 kDa are more resistant to most peptidases and have good bioavailability after inhalation (Niven *et al*., 1994).

Persons with CF have increased activity of serine proteases on the respiratory epithelial surface. Neutrophils, when activated or degenerating, release proteases such as elastase that can directly damage epithelial cells and impair airway clearance. It has been shown that neutrophil proteases cause a secretory response from submucosal glands with an increase in mucus production (Kishioka *et al*., 2001). Intravenous administration, or inhalation, of α_1AT suppresses the activity of neutrophil elastase and restores the bacterial killing capacity of neutrophils. Recombinant secretory leukocyte protease inhibitor (rSLPI) when given to a small number of CF patients at a dose of 100 mg b.i.d. for two weeks decreased neutrophil elastase and IL-8 in airway fluid but was ineffective at a dose of 50 mg twice daily (McElvaney *et al*., 1992). Although this is promising, a number of issues need to be resolved before these or similar agents can be used to prevent damage due to unchecked protease activity in patients with CF.

20.4.3 Fusion protein transport

Active transport mechanisms can facilitate absorption of large molecules across epithelial surfaces. For example, erythropoietin (Epo) is the haematopoetic stimulator of red cell production and differentiation produced in the kidney. Recombinant human Epo is used for the treatment of anaemia associated with chronic renal failure, cancer, and HIV infection. It is administrated chronically by intravenous or subcutaneous injection.

Epo-Fc (molecular weight 112 kDa) is a prototype molecule comprised of human Epo fused to the Fc portion of IgG1. Erythropoietin-Fc fusion protein targets a naturally occurring receptor-mediated transport pathway to deliver Epo systemically via the lung. The usual function of the neonatal Fc receptor or FcRn, is to transport immunoglobulin across cells and protect circulating Ig from degradation. FcRn was first identified in the intestinal epithelium of neonatal rodents, and was shown to bind IgG from mother's milk and transport it across the intestinal epithelium into the circulation, providing temporary immunity in the first weeks of life. These receptors are relatively abundant in adult lung tissues, with greater concentations in the upper and central airway than in the lung periphery. The Fc region of the antibody transports the fusion protein with Epo by receptor-mediated transcytosis via the FcRn receptor in the bronchial and alveolar epithelium. Bronchial airway have more abundant FcRn receptors with less potential for clearance by macrophages (Dellamary *et al*., 2004). Transport of the fusion protein, targeted to the central airway, has been documented in both nonhuman primates and humans. (Dumont *et al*., 2005). This strategy is being used

in the clinical development not only of erythropoietin, but also follicle-stimulating hormone, interferon β and other proteins (Dumont *et al*., 2005).

20.4.4 Gene therapy

Gene therapy involves the vector-mediated transfection of cells lacking a functional target gene with normal complementary DNA (cDNA) for the gene in question, with subsequent activation of the transfected gene and protein production (Thompson and Wiener-Kronsish, 1997). Gene therapy offers possible treatment of both heritable and acquired pulmonary diseases. Efforts to date have largely centred on cDNA transfer of the normal CF transmembrane ion regulator (CFTR) gene to CF patients (Flotte *et al*., 2001). Gene transfer was first attempted by inserting the normal CFTR gene into a replication-defective adenovirus vector with bolus bronchoscopic delivery of the vector. A life-theratening host immune response to the vector led to re-evaluation of this strategy (Knowles *et al*., 1995).

For gene transfer to be effective, the vector and its package must be non-immunogenic, stable to shear forces during aerosolization, and safe to transfected cells. The vector should either stably integrate into the progenitor (basal or stem) cell genome or be safe and effective with repeated administration and should be able to reach the cellular target of relevance. Part of the difficulty with CF is that this cellular target has not been clearly identified as epithelial cell, goblet cell, submucous gland, or all of these. The amount of gene and vector, and its persistence in the airway, must also be determined for each vector and delivery system (Rochat and Morris, 2002).

Virus vectors that have been studied include adenoviruses, adeno-associated virus, and lentivirus. Adenoviruses naturally target the airway epithelium. Adeno-associated viruses are very small organisms that require a 'helper' virus to replicate. These viruses are capable of site-directed insertion into DNA, reducing the risk of insertional mutagenesis (initiating cancer by activation of an oncogene or inactivation of an oncogene suppressor). Gene therapy with the AAV appears to be especially promising (Moss *et al*., 2004), but packing capacity is much smaller with this extremely small virus, making insertion of cDNA cassettes for large genes extremely challenging.

Lentiviruses are retroviruses such as human immunodeficiency virus (HIV). They are able to transfect cells that are not terminally differentiated, such as the basal or airway progenitor cell, but insertional mutagenesis is a substantial risk (Copreni *et al*., 2004).

The primary non-virus vectors studied to date have been cationic liposomes. These lipid capsules are able to form complexes with DNA and then enter cells. With the first generation of liposome vectors, the efficiency of gene transfer was poor; however, this has improved with newer systems (Eastman and Scheule, 1999; Montier *et al*., 2004).

The immune response is a major barrier to gene therapy. The human airway has developed a number of protective strategies against viral vectors. For example, the lack of expression of the native Coxsackie adenovirus (CAR) receptor on the apical surface of airway epithelial cells limits the infectious potential of adenovirus (Montier *et al*., 2004). Transient reduction in cell-to-cell junctions with calcium phosphate co-precepitation allows access of the vector to the basolateral membrane where the CAR receptor is located (Lee *et al.,* 1999).

Mucus and sputum are a significant barrier to gene delivery in the diseased airway. A variety of components of mucus can bind to adenovirus and prevent its association with receptors (West and Rodman, 2001). Gene transfer efficiency through CF sputum is increased when the sputum is pretreated with dornase alfa (Stern *et al*., 1998).

20.4.5 Surfactant phospholipids

Surfactant is produced in the conducting airway as well as in the alveolus and is essential for airway function and cough clearance. A thin surfactant layer between the periciliary fluid and the mucous gel prevents airway dehydration, permits mucus spreading upon extrusion from glands, and allows efficient ciliary coupling with mucus and, more importantly, ciliary release from mucus once kinetic energy is transmitted. There is severe loss of surfactant in the inflamed airway of patients with chronic bronchitis or CF (Griese *et al*., 2005), and surfactant aerosol improves pulmonary function and sputum transportability in patients with chronic bronchitis or CF (Anzueto *et al*., 1997). As a wetting and spreading agent, surfactant also has the ability to increase the lower airway deposition of other aerosol medications, such as dornase alfa or gene therapy vectors, and may increase small particle translocation through the mucus layer (Schürch *et al*., 1990).

20.5 Specific proteins

20.5.1 Insulin

Insulin was one of the first medications to be administered by aerosol (Gaensslen, 1925; Mallet *et al*., 1997). Because of the nebulizer and insulin formulation available at that time, absorption and efficacy were highly unpredictable. This has changed dramatically with the development of ultrafine particles, and of aerosol devices that can efficiently and reliably target the alveolar space. With a rapid and smooth onset of action, and elimination of the necessity for injections with their attendant risks and discomfort, inhaled insulin has great potential for clinical use. Pulmonary insulin administration to healthy subjects can induce hypoglycaemia and a clinically relevant increase in serum insulin concentrations (Jedle and Karlberg, 1996).

Once plasma glucose levels are normalized, postprandial glucose levels can be maintained below diabetic levels by delivering insulin into the lungs 5 minutes before ingestion of a meal (Heinemann *et al*., 1997). Studies have confirmed that inhaled insulin is safe and effective for the therapy of type 2 diabetes, even when this is not controlled by diet (Laube *et al*., 1998; DeFronzo *et al*., 2005), and that the addition of inhaled insulin or oral therapy with hypoglycaemic agents improves glycaemic control (Rosenstock *et al*., 2005).

With the success of inhaled insulin as safe and effective systemic administration of a complex protein via the pulmonary bed, it is highly probable that we see the development of other aerosol therapies that could revolutionize fields as diverse as endocrinology, critical care, immunology, and genetics (Edwards and Dunbar, 2002).

20.5.2 Inhaled immunizations

Since the 1980s, live measles vaccine has been administered by aerosol to 4 million children in Mexico. Studies have shown this to be safe and immunogenic when administered as a first dose at 9–12 months of age (Laube, 2005; Sabin *et al*., 1983). The aerosol is also able to induce mucosal and cellular imunity in older children (>6 years) when administered as a booster dose (Bennett *et al*., 2002). Indeed, aerosolized vaccination with measles, measles–rubella and mumps–measles–rubella vaccine induces better measles antibody

booster responses than injected vaccines (Fernandez de Castro *et al*., 2005). Pulmonary inhalation appears to be superior to nasal administration of live measles vaccine.

Mucosal vaccination may also be superior for non-replicative vaccines which are currently administered parenterally. Immune response and protection afforded by pulmonary administration of inactivated influenza vaccine was demonstrated clinically by Small and colleagues as long ago as 1969 (Waldman *et al*., 1969). Pulmonary administration of the aerosol Fluzone® (Aventis Pasteur, Swiftwater, PA) vaccine was more effective than parenteral or nasal administration in triggering specific immunity, with a more robust humoral and cellular-based immunity local and systemic response (Smith *et al*., 2003). Pulmonary administration of DNA vaccines may also induce a more robust immune response than parenteral administration (Lombry, 2004).

Inhaled vaccines have been used against biowarfare agents (such as anthrax, plague, tularaemia, smallpox) (Alibek, 1999). Similar vaccines are being developed for other agents, with early evidence that aerosol administration may be more effective than parenteral routes (Hassani *et al*., 2004).

The World Health Organization, in collaboration with the Gates Foundation, is currently conducting trials to approve an inhaled live virus measles vaccine for use in third world campaigns. Their goal is to replace injections by inhalation in the vaccination of 300 million children each year (Henoa-Restrepo and Aguado, 2006).

20.5.3 Antibodies

Antibodies administered by intravenous injection must reach high enough concentration at the target organ to achieve therapeutic effect. Pulmonary administration of antibodies can achieve concentrations 10–100-fold higher than injection (Dellamary *et al*., 2004). Therapeutic antibodies are being developed for pulmonary diseases such as cancer, respiratory infections and allergic and inflammatory diseases.

Retentive particles with slow IgG release improve interstitial IgG targeting by avoiding saturation of putative IgG transporters. However, nonretentive particles that release the immunoglobulin rapidly may circumvent particle clearance on the mucociliary escalator and phagocytosis by alveolar macrophages. Concentrations as high as 60 per cent of inhaled IgG dose can be deposited in the interstitial tissue. Although retentive particles were rapidly cleared by alveolar macrophages in an Fc receptor mediated scavenging process, molecules in the interstitium were absorbed over a period of days, acting as a depot of immunoglobulin (Dumont *et al*., 2005). The potential for antibodies to achieve a depot in the pulmonary interstitium may enable sustained delivery of peptides and proteins.

Particulate formulations can enhance immune response to determinants expressed by incorporated proteins. The development of these neutralizing antibodies may trigger unresponsiveness to subsequent administrations. Administration of an intravenous priming dose can reduce this effect (Wolff, 1998).

20.6 Conclusions

An increasing number of novel therapies for pulmonary delivery intended for local or systemic target are in development. Improved understanding of formulations, particle engineering, aerosol delivery, and the pulmonary epithelial barriers combine to improve effective dosing to both local and systemic targets.

References

Alibek K. (1999) *Biohazard: The Chilling Story of the Largest Covert Biological Weapons Program in the World – Told from the Inside by the Man Who Ran It*. Random House, New York.

Anzueto A, Jubran A, Ohar JA, Piquette CA, Rennard SI, Colice G, Pattishall EN, Barret J, Engle M, Perret K, Rubin BK. (1997) Effects of aerosolized surfactant in patients with stable chronic bronchitis. A prospective randomized controlled trial. *J Am Med Assoc* **278**, 1426–1431.

Bennett JV, Fernandez de Castro J, Valdespino-Gomez JL, Garcia-Garcia Mde L, Islas-Romero R, Echaniz-Aviles G, Jimenez-Corona A, Sepulveda-Amor J. (2002) Aerosolized measles and measles–rubella vaccines induce better measles antibody booster responses than injected vaccines: randomized trials in Mexican schoolchildren. *Bull World Health Organ* **80**, 806–812.

Berthiaume Y, Staub NC, Matthay MA. (1987) Beta-adrenergic agonists increase lung liquid clearance in anesthetized sheep. *J Clin Invest* **79**, 335–343.

Blake JR, Sleigh MA. (1974) Mechanics of ciliary locomotion. *Biol Rev Camb Philos Soc* **49**, 85–125

Byron PR, Patton JS. (1994) Drug delivery via the respiratory tract. *J Aerosol Med* **7**, 49–75.

Carrol N, Cooke C, James A. (1997) The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics. *Eur Respir J* **10**, 292–300

Carstairs JR, Nimmo AJ, Barnes PJ. (1985) Autoradiographic visualization of beta-adrenoreceptor subtypes in human lung. *Am Rev Respir Dis* **132**, 541–547.

Chanez P, Bourdin A, Vachier I, Godard P, Bousquet J, Vignola AM. (2004) Effects of inhaled corticosteroids on pathology in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **1**, 184–190.

Copreni E, Penzo M, Carrabino S, Conese M. (2004) Lentivirus-mediated gene transfer to the respiratory epithelium: a promising approach to gene therapy of cystic fibrosis. *Gene Ther* **11**, S67–S75.

DeFronzo RA, Bergenstal RM, Cefalu WT, Pullman J, Lerman S, Bode BW, Phillips LS; Exubera Phase III Study Group. (2005) Efficacy of inhaled insulin in patients with type 2 diabetes not controlled with diet and exercise: a 12-week, randomized, comparative trial. *Diabetes Care* **8**, 1922–1928.

Dellamary L, Smith DJ, Bloom A, Bot S, Guo GR, Deshmuk H, Costello M, Bot A. (2004) Rational design of solid aerosols for immunoglobulin delivery by modulation of aerodynamic and release characteristics. *J Control Rel* **95**, 489–500.

Dumont JA, Bitonti AJ, Clark D, Evans S, Pickford M and Newman SP. (2005) Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway. *J Aerosol Med* **18**, 294–303.

Dunbar C, Scheuch G, Sommerer K, DeLong M, Verma A, Batycky R. (2002) *In vitro* and *in vivo* dose delivery characteristics of large porous particles for inhalation. *Int J Pharm* **245**, 179–189.

Eastman SJ, Scheule RK. (1999) Cationic lipid:cDNA complexes for the treatment of cystic fibrosis. *Curr Opin Mol Ther* **1**, 186–196,

Edsbacker S. (2002) Uptake, retention and biotransformation of corticosteroids in the lung and airways. In: *Inhaled Steroids in Asthma. Optimizing Effects in the Airways*. Schleimer RP, O'Byrne PM, Szefler SJ, Brattsand R (eds). Marcel Dekker, New York, pp. 213–246.

Edwards DA, Dunbar C. (2002) Bioengineering of therapeutic aerosols. *Annu Rev Biomed Eng* **4**, 93–107.

Fernandez de Castro J, Bennett JV, Rincon HG, Munoz MT, Sanchez LT, Santos JI. (2005) Evaluation of immunogenicity and side effects of triple viral vaccine (MMR) in adults, given by two routes: subcutaneous and respiratory (aerosol). *Vaccine* **23**, 1079–1084.

Flotte TR, Laube BL. (2001) Gene therapy in cystic fibrosis. *Chest* **120**, 124S-131-S.

Folkesson HG, Matthey MA, Westrom BR, Kim KJ, Karlsson BW, Hastings RH. (1996) Alveolar epithelial clearance of protein. *J Appl Physiol* **80**, 1431–1445.

Forges B, Wilson CG, Gumbleton M. (1999) Temporal dependence of extopeptidase expression in alveolar epithelial cell culture: implications for study of peptide absorption. *Int J Pharm* **180**, 225–234.

Gaensslen M. (1925) Uber inhalation von Insulin. *Klin Wochenschr* **2**, 71–72.

Griese M, Essl R, Schmidt R, Ballmann M, Paul K, Rietschel E, Ratjen F. (2005) Sequential analysis of surfactant, lung function and inflammation in cystic fibrosis patients. *Respir Res* **6**, 133–143.

Groneberg DA, Witt C, Wagner U, Chang KF and Fischer A. (2003) Fundamentals of pulmonary delivery. *Respir Med* **97**, 382–387.

Hassani M, Patel MC, Pirofski LA. (2004) Vaccines for the prevention of diseases caused by potential bioweapons. *Clin Immunol* **111**, 1–15.

Heinemann L, Traut T, Heise T. (1997) Time–action profile of inhaled insulin. *Diabet Med* **14**, 63–67.

Henao-Restrepo AM, Aguado MT. (2006) Measles aerosol immunization and its potential contribution to further reduce measles mortality worldwide. *Respir Drug Deliv* **1**, 65–72.

Holter JF, Weiland JE, Pacht ER, Gadek JE, Davis WB. (1986) Protein permeability on the adult respiratory distress syndrome. Loss of size selectivity of alveolar epithelium. *J Clin Invest* **78**, 1513–1522.

Hussain A, Majumder QH, Ahsan H. (2006) Inhaled insulin is better absorbed when administered as a dry powder compared to solution in the presence or absence of alkylglycosides. *Pharm Res* **23**, 138–147.

Illum L, Fischer AN. (1997) Intranasal delivery of peptides and proteins. In: *Inhalation Delivery of Therapeutic Peptides and Proteins*, Adjei AL, Gupta PK (eds). Marcel Dekker, New York, pp. 135–184.

Inoue S, Michel RP, Hogg JC. (1976) Zonulae occludentes in alveolar epithelium and capillary endothelium of dog lungs studies with the freeze-fracture technique. *J Ultrastruct Res* **56**, 215–225.

Jedle JH, Karlberg BE. (1996) Intrapulmonary administration of insulin to healthy volunteers. *J Int Med* **240**, 93–98.

Kim K-J, Malik AB. (2002) Protein transport across the lung epithelial barrier. *Am J Physiol Lung Cell Mol Physiol* **284**, L247–L259.

Kim D, Mudaliar S, Chinnapongse S, Chu N, Boies SM, Davis T, Perera AD, Fishman RS, Shapiro DA, Henry R. (2003) Dose–response relationships of inhaled insulin delivered via the Aerodose insulin inhaler and subcutaneously injected insulin in patients with type 2 diabetes. *Diabetes Care* **26**, 2842–2847.

Kishioka C, Okamoto K, Kim JS, Rubin BK. (2001) Regulation of secretion from mucous and serous cells in the excised ferret trachea. *Resp Physiol* **126**, 163–171.

Knowles MR, Hohneker KW, Zhou Z, Olsen JC, Noah TL, Hu PC, Leigh MW, Engelhardt JF, Edwards LJ, Jones KR, Grossman M, Wilson JM, Johnson LG, Boucher RC. (1995) A controlled study of adenovirus-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. *N Engl J Med* **333**, 823–831.

Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. (1996) Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* **154**, 1505–1510.

Kreyling WG, Semmler-Behnke M, Moller W. (2006) Ultrafine particle–lung interactions: does size matter? *J Aerosol Med* **19**, 74–84.

Laube BL. (2005) The expanding role of aerosols in systemic drug delivery, gene therapy, and vaccination. *Respir Care* **50**, 1161–76.

Laube BL, Benedict GW, Dobs AS. (1998) The lung as an alternative route for delivery for insulin in controlling postprandial glucose levels in patients with diabetes. *Chest* **114**, 1734–1739.

Lauweryns JM, Baert JH. (1977) Alveolar clearance and the role of the pulmonary lymphatics. *Am Rev Respir Dis* **115**, 625–683.

Lee JH, Zabner J, Welsh MJ. (1999) Delivery of an adenovirus vector in a calcium phosphate coprecepitate enhances the therapeutic index of gene transfer to airway epithelia. *Hum Gene Ther* **10**, 603–613.

Lombry C, Marteleur A, Arras M, Lison D, Louahed J, Renauld JC, Preat V, Vanbever R. (2004) Local and systemic immune responses to intratracheal instillation of antigen and DNA vaccines in mice. *Pharm Res* **21**, 127–135.

Mak JCW. Barnes PJ. (1990) Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. *Am Rev Respir Dis* **141**, 1559–1568.

Mallet JP, Diot P, Lemarie E. (1997) Inhalation route for administration of systemic drugs. *Rev Malad Respir* **14**, 257–267.

Matsukawa Y, Yamahara H, Lee VH, Crandall ED, Kim KJ. (1996) Horseradish peroxidase transport across rate alveolar epithelial cell monolayers. *Pharm Res* **13**, 1331–1335.

McElvaney NG, Nakamura H, Birrer P, Hebert CA, Wong WL, Alphonso M, Baker JB, Catalano MA, Crystal RG. (1992) Modulation of airway inflammation in cystic fibrosis: *in vivo* suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor, *J Clin Invest* **90**, 1296–1301.

Montier T, Delepine P, Pichon C, Ferec C, Porteous DJ, Midoux P. (2004) Non-viral vectors in cystic fibrosis gene therapy: progress and challenges. *Trends Biotechnol* **22**, 586–592.

Moss RB, Rodman D, Spencer LT, Aiken ML, Zeitlin PL, Waltz D, Milla C, Brody AS, Clancy JP, Ramsey B, Hamblett N, Heald AE. (2004) Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* **125**, 509–521.

Newhouse MT, Corkery KJ. (2001) Aerosols for systemic delivery of macromolecules. *Respir Care Clin N Am.* **7**, 261–275.

Niven RW, Lott FD, Ip AY, Cribbs JM. (1994) Pulmonary delivery of powders and solutions containing recombinant human granulocyte colony-stimulating factor (rhG-CSF) to the rabbit. *Pharm Res* **11**, 1101–1109.

Oberdorster G, Oberdorster E, Oberdorster J. (2005) Nanotoxicity: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* **113**, 823–839.

Okamoto H, Todo H, Iida K, Danjo K. (2002) Dry powders for pulmonary delivery of peptides and proteins. *KONA* **20**, 71–83.

Panettari RJ, Jr. (2004) Effects of corticosteroids on structural cells in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **1**, 231–234.

Patton JS. (1996) Mechanisms of macromolecule absorption by the lungs. *Adv Drug Delivery Rev* **19**, 3–36.

Ralston E, Ploug T. (1999) Caveolin-3 is associated with the T-tubules of mature skeletal muscle fibers. *Exp Cell Res* **246**, 510–515.

Rathberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG. (1992) Caveolin, a protein component of of caveolae membrane coats. *Cell* **68**, 673–682.

Rochat T, Morris MA. (2002) Gene therapy for cystic fibrosis by means of aerosol. *J Aerosol Med* **15**, 229–235.

Rosenstock J, Zinman B, Murphy LJ, Clement SC, Moore P, Bowering CK, Hendler R, Lan SP, Cefalu WT. (2005) Inhaled insulin improves glycemic control when substituted for or added to oral combination therapy in type 2 diabetes: a randomized, controlled trial. *Ann Intern Med* **143**, 549–558.

Ruffin RE, Dolovich MB, Wolff RK, Newhouse MT. (1978) The effects of preferential deposition of histamine in the human airway. *Am Rev Respir Dis* **117**, 485–492.

Sabin AB, Flores Arechiga A, Fernandez de Castro J, Sever JL, Madden DL, Shekarchi I, Albrecht P. (1983) Successful immunization of children with and without maternal antibody by aerosolized measles vaccine I. Different results with human diploid cell and chick embryo fibroblast vaccines. *JAMA* **249**, 2651–2662.

Schnitzer JE, Liu J, Oh P. (1995) Endothelial caveolae have the molecular transport machinery for vesicle budding, docking and fusion including VAMP, NSF, SNAP, annexins, and GTPases. *J Biol Chem* **270**, 14299–14404.

Schürch S, Gehr P, Im Hof V, Geiser M, Green F. (1990) Surfactant displaces particles toward the epithelium in airways and alveali. *Respir Physiol* **80**, 17–32.

Serafini SM, Michaelson ED. (1977) Length and distribution of cilia in human and canine airways. *Bull Eur Physiopathol Respir* **13**, 551–559.

Sibile Y, Reynolds HY. (1990) Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* **141**, 471–501.

Smith DJ, Bot S, Dellamary L, Bot A. (2003) Evaluation of novel aerosol formulations designed for mucosal vaccination against influenza virus. *Vaccine* **21**, 2805–2812.

Sorokin SP. (1970) Properties of alveolar cells and tissues that strengthen alveolar defenses. *Arch Intern Med* **126**, 450–463.

Stern M, Caplen NJ, Browning JE, Griesenbach U, Sorgi F, Huang L, Gruenert DC, Marriot C, Crystal RG, Geddes DM, Alton EW. (1998) The effect of mucolytic agents on gene transfer across a CF sputum barrier *in vitro. Gene Ther* **5**, 91–98.

Stone KC, Mercer RR, Gehr P, Stockstill B, Crapo JD. (1992) Allometric relationship of cell numbers and size in the mammalian lung. *Am J Respir Cell Mol Biol* **6**, 235–243.

Stossel TP. (1977) Phagocytosis. Clinical disorder of recognition and ingestion. *Am J Pathol* **88**, 741–51.

Summers QA. (1991) Inhaled drugs and the lung. *Clin Exp Allergy* **21**, 259–268.

Taylor AE, Gaar KAA, Jr. (1970) Estimation of equivalent pore radii of pulmonary capillary and alveolar membranes. *Am J Physiol* **218**, 1133–1140.

Thompson MM and Wiener-Kronish JP. (1997) General issues in gene delivery via the lung. In: *Inhalation Delivery of Therapeutic Peptides and Proteins*, Adjei AL, Gupta PK (eds). Marcel Dekker, New York.

Usmani OS, Biddiscombe MF, Barnes PJ. (2005) Regional lung deposition and bronchodilator response as a function of beta2-agonist particle size. *Am J Respir Crit Care Med* **172**, 1497–504.

Waldman RH, Mann JJ, Small PA. (1969) Immunization against influenza: prevention of illness in man by aerosolized inactivated vaccine. *JAMA* **207**, 520–524.

West J, Rodman DM. (2001) Gene therapy for pulmonary diseases. *Chest* **119**, 613–617.

Wolff RK. (1998) Safety of inhaled proteins for therapeutic use. *J Aerosol Med* **11**, 197–219.

Yamada K, Odomi M, Okada N, Fujita T, Yamamoto A. (2005) Chitosan oligomers as potential and safe absorption enhancers for improving the pulmonary absorption of interferon-α in rats. *J Pharm Sci* **94**, 2432–2440.

Yang T, Mustafa F, Bai S, Ahsan F. (2004) Pulmonary delivery of low molecular weight heparins. *Pharm Res* **21**, 2009–2016.

Index

Note: page numbers in *italics* refer to figures or tables

The Pulmonary Epithelium in Health and Disease Edited by David Proud

ISBN: 978-0-470-05951-7