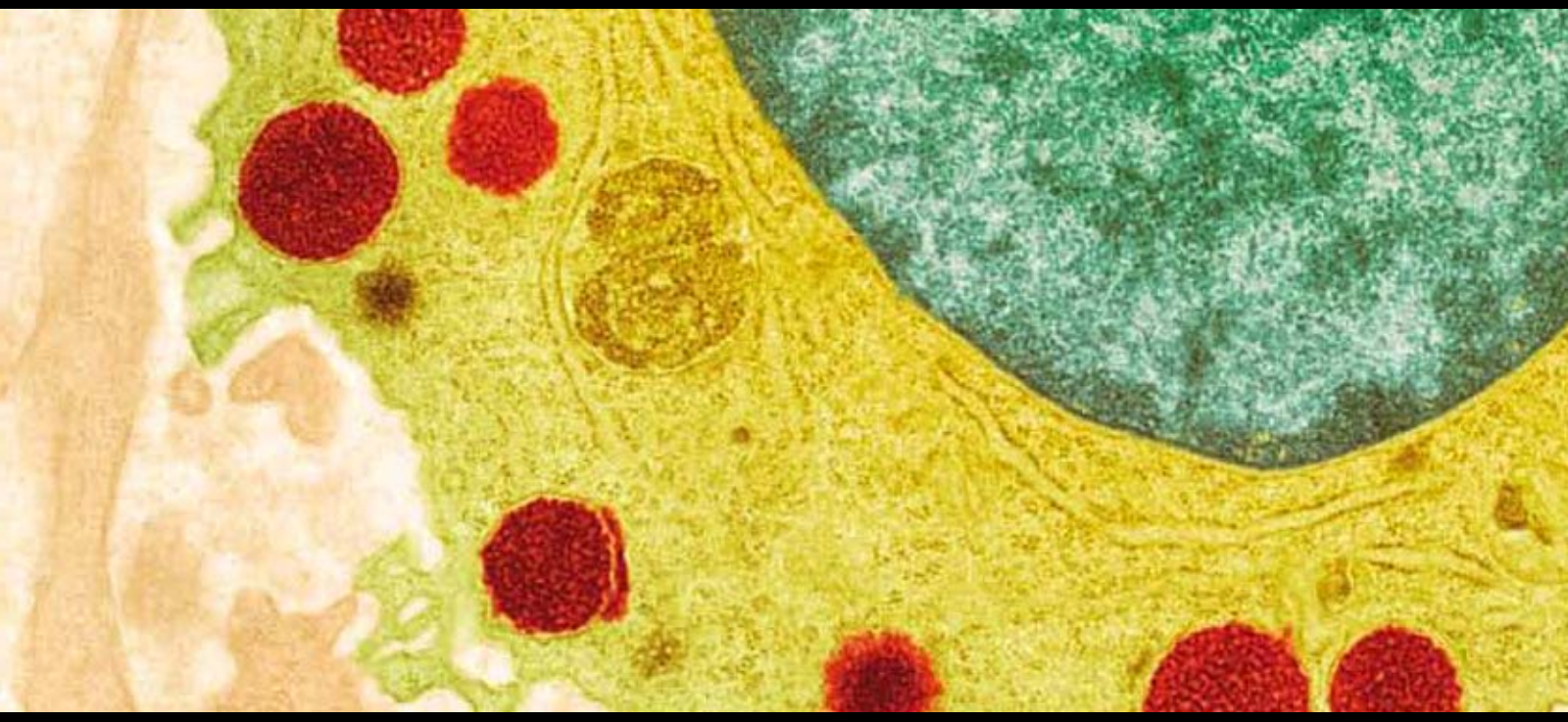
Journal of Allergy

Function of the Airway Epithelium in Asthma

Guest Editors: Teal S. Hallstrand, Prescott G. Woodruff, Stephen T. Holgate, and Darryl A. Knight



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Editorial Board

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Editorial

Function of the Airway Epithelium in Asthma

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An increasingly strong body of evidence implicates the airway epithelium as a critical regulator of airway inflammation and remodeling relevant to the pathogenesis of asthma. The evidence implicating the epithelium includes *in vivo* studies in humans and in murine models as well as *in vitro* studies conducted with primary epithelial cells. The purpose of this special issue is to provide a forum to integrate new knowledge about the nature of epithelial dysfunction in asthma and examine the role of the epithelium as a regulator of immune function. The articles contained within this special issue expound upon human studies examining epithelial gene expression, alterations in epithelial structure, as well as *in vitro* studies investigating epithelial function relevant to asthma.

This special issue contains 3 original research articles and 4 review articles including some that delineate larger bodies of work by well-established research labs. The review articles focus on mechanisms related to the epithelium including the response to fungal allergens, epithelial apoptosis, production of mediators involved in remodeling of the matrix, and the important role of epithelial injury and the production of IL-25 in the induction phase of asthma. Two of the research articles highlight pathways of epithelial activation leading to inflammation that diverges from the traditional Th2 paradigm. In the final research article, the authors examine the utility of *in vitro* epithelial models.

In the paper entitled "*Mechanisms of remodeling in asthmatic airways,*" A. Shifren et al. from the group at Washington University including Dr. Mario Castro and others present an overview of structural changes that occur in patients with asthma and are particularly prominent in patients with severe asthma. In particular, their review outlines several mechanisms of epithelial activation leading to these structural changes in the subepithelial matrix as well as mucous cell alterations leading to further airflow obstruction. The roles of various therapies that may target remodeling are discussed.

In the paper "*Apoptosis and the airway epithelium*," S. R. White from the University of Chicago presents a thorough overview of the function of epithelial apoptosis in health and disease states focusing on asthma, but also extending to other pulmonary diseases. The article has an outstanding overview of the function of apoptosis in the epithelium in contrast to immune cells and the basic mechanisms that regulate apoptosis in the epithelium. The article clearly delineates alterations in apoptosis that have been identified in human studies and *in vivo* animal models and the reasons that these alterations in the epithelium may play a pathogenic role in asthma and other lung diseases involving the epithelium.

In the paper "*Responses of airway epithelium to environmental injury: role in the induction phase of childhood asthma*," R. K. Kumar and others from the University of New South Wales along with P. Foster and others from the University of Newcastle present a detailed picture of the factors involving the epithelium that may initiate airway inflammation. In particular, the response of the epithelium

to viral infection and environmental pollutants such as diesel exhaust particles are presented as common triggers that occur in the epithelium leading to the generation of important epithelial factors including interleukin 25 (IL-25) and thymic stromal lymphopoietin (TSLP). The authors present evidence from their own work and others using studies in epithelial model systems as well as *in vivo* studies.

The final review article "*Immunopathology and immunogenetics of allergic bronchopulmonary aspergillosis*," was written by A. P. Knutsen from the Department of Pediatrics at Saint Louis University. The review focuses on the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA), a disease characterized by fungal hypersensitivity associated with central bronchiectasis and markedly elevated IgE. By relating a significant body of work relating to the epithelial response to aspergillus and other mold antigens, the author presents compelling evidence that the epithelium plays a key role in the pathogenesis of ABPA by regulating lymphocyte trafficking and activation.

In the original research article titled, "*Immunolocalization of NLRP3 inflammasome in normal murine airway epithelium and changes following induction of ovalbumin-induced airway inflammation*," H. B. Tran et al. from the University of Adelaide use the murine model of ovalbumin-induced airway inflammation to examine the activation of the NLRP3 inflammasome in the airway epithelium. Little is known about the activation of this aspect of the innate immune system in asthma or model systems of asthma. In the epithelium, the authors found evidence of active caspase-1 and a redistribution of caspase-1, IL-1β, and IL 18 towards the luminal surface following sensitization and challenge with ovalbumin. These intriguing results should spur more research into triggers of asthma that may lead to inflammasome activation and the specific role of the inflammasome in models of asthma.

In the research paper "*IL-17F induces CCL20 in bronchial epithelial cells*," K. Nozato et al. examine the regulation of CCL20 expression by IL-17F in bronchial epithelial cells. The authors demonstrate that IL-17F modulates CCL20 expression through a mitogen-activated protein kinase (MAPK) pathway leading to the activation of the cyclic AMP response element-binding (CREB) transcription factor. This paper adds to other evidence by this investigative team demonstrating that IL-17F induces CXC chemokines, GM-CSF, IP-10, IL-11, and IGF-1 through this pathway.

In the final research paper "*Evaluation of differentiated human bronchial epithelial cell culture systems for asthma research*," C. F. Stewart et al. from the University of Nottingham working with I. Sayers evaluated differences between *in vitro* models of the epithelium. The investigators used primary human bronchial epithelial cells as well as several cell lines including a lung adenocarcinoma cells line (Calu-3) and SV-40-transformed human bronchial epithelial cells (BEAS-2B). Using these cells they found that there were differences in markers of differentiation such as mucous cell differentiation between the cultured cells, as well as differences between the junction proteins. Although the basis of the differences are is know in detail, the research found that there were differences between primary epithelial cells donor as well as between cultures that originated from the same donor raising important questions about the variability of these model systems.

Taken together, these articles provide yet further evidence that in asthma the epithelium takes center stage in orchestrating responses to the inhaled environment through pathways influencing inflammation as well as aberrant repair. In addition, the epithelium provides a platform for identifying novel therapeutic targets linked to increasing the resistance of the airways to environmental injury. We are exceedingly grateful to the many leaders in the field of epithelial biology and asthma who have contributed to this special issue. The strong response to this call for papers by well-recognized researchers in this field provides additional evidence of the emerging importance of the field of epithelial biology to the understanding of asthma.

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Research Article

Immunolocalization of NLRP3 Inflammasome in Normal Murine Airway Epithelium and Changes following Induction of Ovalbumin-Induced Airway Inflammation

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Little is known about innate immunity and components of inflammasomes in airway epithelium. This study evaluated immunohistological evidence for NLRP3 inflammasomes in normal and inflamed murine (Balb/c) airway epithelium in a model of ovalbumin (OVA) induced allergic airway inflammation. The airway epithelium of control mice exhibited strong cytoplasmic staining for total caspase-1, ASC, and NLRP3, whereas the OVA mice exhibited strong staining for active caspase-1, with redistribution of caspase-1, IL-1β and IL-18, indicating possible activation of the NLRP3 inflammasome. Active caspase-1, NLRP3, and other inflammasome components were also detected in tissue eosinophils from OVA mice, and may potentially contribute to IL-1β and IL-18 production. In whole lung, inRNA expression of NAIP and procaspase-1 was increased in OVA mice, whereas NLRP3, IL-1β and IL-18 decreased. Some OVA-treated mice also had significantly elevated and tightly correlated serum levels of IL-1β and TNFα. In cultured normal human bronchial epithelial cells, LPS priming resulted in a significant increase in NLRP3 and II-lp protein expression. This study is the first to demonstrate NLRP3 inflammasome components in normal airway epithelium and changes with inflammation. We propose activation and/or luminal release of the inflammasome is a feature of allergic airway inflammation which may contribute to disease pathogenesis.

1. Introduction

Asthma affects up to 12% of adults and 25% of children in Australia and there is a significant undiagnosed cohort [1]. Although we have a range of medications that are effective in their own right, there is a need to further improve the management of this disease. This involves better clinical programs and an improved understanding of the basic mechanisms of asthma so that new ways can be introduced which work synergistically with conventional asthma medications to modify airway inflammation.

Asthma is a disease characterized by both bronchiolar smooth muscle constriction and a chronic airway inflammation. Some of the features of the disease are modelled in

the well-characterized acute and chronic models of OVA-induced allergic airway inflammation in mice [2, 3]. The inflammatory component of asthma is generally thought of as a Th2-driven process, involving eosinophil recruitment to the airways and consequent damage, including airway epithelial cell death [4]. Damage and repair eventually lead to a remodelling of the airways which increases the sensitivity of the airway muscle to cholinergic agonists and further restricts airflow.

However, other inflammatory signalling pathways may also play a significant role in the pathogenesis and progression of the disease. Of particular interest is the role of the IL-1 family of cytokines that are key components of the innate immune response [5]. IL-1β and IL-18 are activated by cytosolic multiprotein complexes called inflammasomes [6, 7]. Inflammasomes have been best characterized in the monocyte-macrophage cell lineage, but recent evidence indicates that gingival (and perhaps other) types of epithelial cells may also contain these structures [8]. Their generic structure includes (i) a member of the nucleotide-binding oligomerization domain- (NOD-) like receptor (NLR) family of pattern recognition molecules specific for each type of inflammasome, (ii) apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and (iii) caspase-1. Other inflammatory caspases or caspase-regulatory molecules such as X-linked or neuronal inhibitor of apoptosis proteins, XIAP and NIAP, respectively, may also be recruited. Different types of inflammasome (e.g., Nlrp1, Nlrp3, IPAF, and AIM2) have been identified based on the NLR component (or the non-NLR equivalent) which forms the complex. Of these the best characterized is the Nlrp3 inflammasome which plays a predominant role in IL-1β and IL-18 production [7]. In macrophages, IL-1β production, processing, and release requires the interaction of two signalling pathways. Binding of ligands such as lipopolysaccharide (LPS) to membrane toll-like receptor 4 (TLR-4) triggers the synthesis of pro-IL-1β while a number of danger signals including molecules released from necrotic cells (e.g., ATP and uric acid) promote assembly of the Nlrp3 inflammasome complex, activation of caspase-1 from its precursor, processing of IL-1β to its active form, and release of IL-1β from the cells [8].

Inflammasome involvement in asthma inflammation is a relatively new concept. An early protective role for inflammasomes might be predicted by the "hygiene hypothesis", whereby exposure to microbes and their products (such as LPS) early in life is thought to protect against development of asthma, perhaps by a skewing of the immune response away from one dominated by Th2 cytokines [9]. However, current evidence would favour a proinflammatory role for IL-1β since (i) there are increased levels of serum, BALF, and bronchial epithelial IL-1β in human asthmatics, compared to healthy subjects [10–12], (ii) increase in serum IL-1β has also been reported in primates [13], (iii) IL-1β levels were decreased 2-fold in the bronchial epithelium following inhalation of beclomethasone dipropionate (as measured by an immunohistological technique) [14], and (iv) administration of TNF-α and IL-1β induces airway hyperreactivity, a feature of asthma [15, 16]. IL-18, another potent pro-inflammatory cytokine whose maturation requires activation of caspase-1 on the inflammasome, is typically considered as a Th1 cytokine due to its effects associated with IFN-γ. Increased serum IL-18 has also been described in asthmatics [17–19]. Finally, danger signal molecules such as uric acid and extracellular ATP have been shown to mediate inflammasome-dependent inflammation in experimental rodent models of lung injury [20] and asthma [21], respectively.

In addition to providing a physical barrier to inhaled pathogens, allergens, and other foreign agents, the airway mucosa (epithelium and secretions) has a number of potential mechanisms by which it can contribute to innate immune defences in the lung. Loss of the integrity of the airway epithelium is widely thought to be a critical component in the pathogenesis of asthma [22]. To our knowledge, there has been no systematic study of the inflammasome in airway epithelium. In this study, we have looked for evidence of Nlrp3 inflammasome involvement in OVA-induced airway inflammation in mice, as a model of human asthma, as well as in primary bronchial epithelial cultures.

2. Materials and Methods

2.1. Antibodies. Rabbit polyclonal antibodies (Abs) to IL-1β, Nlrp3, total caspase-1; goat Abs to caspase-1 P10 and P20 active subunits; and mouse monoclonal Ab (mAb) to SP-D were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other rabbit Abs were ASC from Abcam (Cambridge, UK), CCR3 from Epitomics (Burlingame, CL, USA), and IL-18 from Rockland (Gilbertsville, Pennsylvania, USA). Mouse mAb to human NLRP3 was from Enzo (Enzo Life Sciences Inc., Farmingdale, NY, USA). Secondary Abs were sheep F(ab′)2 anti-rabbit IgG (Cy3), goat F(ab′)2 anti-rabbit IgG (FITC), rabbit anti-goat IgG (Cy3), sheep F(ab′)2 anti-mouse IgG (Cy3), (Sigma-Aldrich Chemicals, St Louis, MO, USA), and a donkey F(ab′)2 anti-mouse IgG DyLight594 (Jackson ImmunoResearch, West Grove, PA, USA).

2.2. Mouse Model of Allergic Airway Inflammation. A classical mouse model [23] that induces strong eosinophilic inflammation, airway hyper-responsiveness, and vascular and parenchymal changes after sensitisation and challenge with OVA was used to induce inflammation. All experiments were performed under the University of Adelaide Animal Ethics Committee approval number M-57-2007, and in compliance with "Principles of Animal Care" publication number 86-23 of the National Institute of Health and the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes", 6th Edition.

Female Balb/c mice (age 4–6 weeks; specific pathogen free) were purchased from the University of Adelaide, Adelaide, Australia. Three mice were set aside for analysis of inflammasome components in the absence of any stimulus. The remaining mice were divided into two experimental groups ($n = 8$ in each group) and housed in plastic cages (38×25 cm) at 21°C with a 14 h light/10-h dark cycle.

OVA-treated mice received 50 μg of chicken OVA in 1 mL of alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline, i.p. on days 0 and 14. Control (SAL) mice received alhydrogel in 0.9% saline alone. Mice sensitized to OVA were then aerochallenged with 10 mg/mL OVA in 0.9% saline from day 22 to day 32, for 30 min, three times a day, every 2nd day, using a side-stream nebulizer, which produced particles of 1–3 μm (Fisher and Paykel, Sydney, Australia). SAL-treated groups were nebulised with 0.9% saline alone.

2.3. Collection of Tissues. Mice were bled from the tail for preparation of sera. For tissue collection, mice were anaesthetized by an i.p. dose of pentobarbitone sodium (50 mg kg^{-1}), 22 h after the last nebulization. The trachea was cannulated with a blunted 19-gauge needle and bronchoalveolar lavage fluid (BALF) was collected by lavaging the lungs three times by instilling and withdrawing the same volume (1 mL) of ice-cold Hanks buffered salt solution (HBSS, pH 7.4). One part of the BALF sample was immediately cytospun onto slides and the other part was centrifuged to obtain supernatant for cytokine and uric acid assay. Mice were then sacrificed and other tissue samples collected. Tissue samples were archived as appropriate for various downstream measurements, including standard assessments of airway and tissue eosinophilia and lung histopathology.

2.4. Isolation of Lung mRNA and Reverse Transcription. Samples of mouse lung tissue were immediately placed into RNA later (Applied Biosystems/Ambion, USA). Tissue was homogenized using a TissueRuptor (Qiagen Pty Ltd, Australia) and RNA extracted using an RNeasy Mini Kit (Qiagen Pty Ltd, Australia). An on-column DNAse treatment was performed using an RNase-Free DNase Set (Qiagen Pty Ltd, Australia). RNA quality was checked using an Experion RNA StdSens Kit on the Experion Electrophoresis station (BioRad Laboratories Inc, Australia). Mouse RNA samples obtaining RQI of 7–10 (green) were used in the subsequent array analysis. RNA content was quantified using a Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA). 1000 ng of RNA was reverse transcribed into cDNA using the RT^2 First Strand Kit (Qiagen Pty Ltd, Australia). The incubation steps for the reaction were performed on a MyCycler Thermal Cycler (BioRad Laboratories Inc, Australia).

2.5. Real-Time Quantitative PCR Array. Mouse cDNA samples were combined with RT^2 SYBR Green/Fluorescein qPCR Master Mix with Nuclease-free Sterile H_2O (Qiagen Pty Ltd, Australia) and then added across an entire RT2 Profiler PCR Mouse Inflammasome Array (PAMM-097A) 96-well plate. Each mouse sample ($n = 8$ from each group) was tested on a separate 96-well array plate containing a panel of 84 wells pertaining to different genes of the inflammasome pathway, 5 house keeping control wells, 1 genomic DNA control well, 3 reverse transcription control wells, and three positive PCR control wells. PCR was performed using an iCycler with iQ5 Software (BioRad Laboratories Inc, Australia) and Cq data was then analysed by the $\Delta\Delta$Ct method using the RT^2 Profilier Array data spreadsheet (Qiagen Pty Ltd, Australia).

2.6. Measurement of Cytokines. Serum cytokines were measured by Bioplex as per the manufacturer's instructions (Bio-Rad Laboratories Inc, Australia). In brief, the concentration of total protein per sample was determined by Mini Bradford assay (BioRad Laboratories Inc, Australia) using standards of bovine serum albumin (Sigma-Aldrich Chemicals, St Louis, MO, USA). Samples were normalised to 100 μg total protein and analysed in duplicate using a Bioplex Promouse Cytokine 8 plex magnetic bead array. All wash steps were performed using the Bioplex II Wash Station. Plate data was collected using a Bioplex 200 suspension array system and analysed using the Bioplex Manager 5 Software (BioRad Laboratories Inc, Australia).

2.7. Immunofluorescence Studies. Paraffin tissue sections of 5 μm thickness were treated for antigen retrieval by microwave heating in 10 mM citrate buffer pH 6.0 unless otherwise stated. Tissue sections were also heated in 10 mM Tris EDTA buffer pH 9.0 for costaining of SP-D with caspase-1, and digested with 1 mg/mL Proteinase K (Promega, Fitchburg, Wisconsin, USA) for staining of P10 and P20 caspase-1 subunits. Sections were blocked with a serum-free protein blocking solution (DakoCytomation Inc., Carpinteria, CA, USA), incubated overnight at 4°C with primary Abs, and 1 h at room temperature with secondary Abs. Immunofluorescence was detected and imaged with a Zeiss microscope equipped with HBO 100 illuminating system, AxioCam MRn digital camera and AxioVision 4.8.1 software (Carl Zeiss GmbH, Goettingen, Germany).

2.8. TUNEL Staining of Cell Death. Cell death was detected in situ using a fluorescence TUNEL kit from Promega (Fitchburg, Wisconsin, USA) following the manufacturer's protocol.

2.9. Measurement of Uric Acid in BALF. Uric acid was measured in BALF according to manufacturer's instructions using the Amplex Red Uric Acid/Uricase Assay Kit (A22181, Invitrogen Aust Pty Ltd, Mulgrave, Australia). Fluorescence measurements were made using a fluorescence plate reader (Optima Fluostar, BMG Labtech, Gmbh, Offenburg, Germany) with excitation at 530 nm and emission read at 590 nm.

2.10. Normal Human Bronchial Epithelial Cell Culture. Normal human bronchial epithelial (NHBE) cells and Bronchial Epithelial Growth Medium (BEGM) were obtained from Lonza (Lonza Australia Pty Ltd, Mt Waverley, VIC, Australia). Cells were cultured in BEGM until use at passage 4. Cells at 60–70% confluence were stimulated for 18 h with *E. coli* LPS (5 μg/mL, Sigma-Aldrich Chemicals, St Louis, MO, USA), IFN-γ (50 ng/mL, Sigma-Aldrich Chemicals, St Louis, MO, USA), or combination of these two, in 8-well chamber slides (BD Biosciences, Franklin Lakes, NJ, USA). Cells were washed twice with phosphate-buffered saline (PBS) and then fixed with 2.5% formalin/PBS for 10 minutes. Cells were washed 4 times with TBST (tris-buffered saline, pH7.5 added with 0.05% Tween-20), air-dried overnight at room temperature, and stored at −20°C until use.

2.11. Immunofluorescence of NHBE. Fixed cells were permeabilized 5 minutes with 1% SDS/PBS at room temperature, then washed 5 times with TBST to remove SDS. Following 1 h blocking incubation with a serum-free protein blocker (DakoCytomation Inc., Carpinteria, CA, USA), cells were incubated with a mixture of primary antibodies (see antibody section of methods) including mouse monoclonal antibody to human Nlrp3 and a rabbit polyclonal anti-human IL-1β, overnight at 4°C. Next, following 5 washes with TBST, cells were incubated 1 h at room temperature with a mixture of secondary antibodies including a donkey F(ab′)2 anti-mouse IgG DyLight594 and goat F(ab′)2 anti-rabbit IgG-FITC. Slides were washed 5 times with TBST before coverslipping and imaging.

For quantitation of fluorescence intensity, multiple microphotos were acquired randomly under a 20x objective. Images were then blinded and analysed using a morphometric software package (ImageJ, NIH, Bethesda, MD, USA).

3. Results

3.1. Parameters of Airway Inflammation. In keeping with previous descriptions of the acute allergic airway inflammation model [2, 23], the airway inflammation in OVA-challenged mice was characterized by dense peribronchial and perivascular infiltrates of leucocytes (mostly eosinophils) and oedema of bronchial epithelium. Epithelial swelling was more prominent in large bronchi that were sometimes occluded by thickened epithelium. There was swelling of endothelial cells in some blood vessels. No increase of mast cells was detected. None of the saline- (SAL-) treated mice showed any notable histological changes. Typically, there was a 7-fold increase in total BALF cell count from $15.6 \pm 11.1 \times 10^4$ (median ± 2.5% CI) cells/mL in SAL controls to $117.7 \pm 82.7 \times 10^4$ cells/mL in OVA-treated mice ($P < 0.0001$). This was largely due to eosinophilia (>85%), although numbers of neutrophils, monocytes, and lymphocytes were also significantly increased ($P < 0.05$, data not shown).

3.2. Presence of Inflammasome Components in Normal Mouse Airway Epithelium. To test the hypothesis that the normal airway epithelium possesses the ability to assemble inflammasome complexes, we used immunofluorescence to analyse formalin-fixed lung tissue sections obtained from healthy Balb/c mice for expression of inflammasome common components Nlrp3, ASC and caspase-1, and the substrate cytokines IL-1β and IL-18. The same results were obtained for completely untreated mice (naive mice, $n = 3$) and SAL mice ($n = 8$) that were sham-treated with saline i.p. injections and saline nebulisation and used as controls in the experimental murine airway inflammation model.

Serial sections of control mouse lungs ($n = 8$) were examined by immunohistological labelling for caspase-1 and various other inflammasome-related proteins. Figures 1(a)–1(c) show colocalization of caspase-1 and Nlrp3 in the epithelia of three bronchioles. The fluorescence staining for caspase-1 could be specifically blocked with the relevant immunogen peptide (Figure 1(c)). Blocking peptides were not available for the other antibodies in this panel. However, omission of primary antibody or section incubation with normal rabbit IgG (at matched IgG concentrations) resulted in negligible fluorescence (Figure 1(f)). Colocalization of caspase-1 and Nlrp3 with IL-1β, IL-18, and ASC in bronchiolar epithelia was shown (Figures 1(d)–1(k)). There was also colocalization in certain cells scattered among alveoli. It should be noted that the antibodies to caspase-1, IL-1β, and IL-18 did not distinguish between their precursor and mature forms. After performing immunofluorescence, some sections were washed and restained with hematoxylin and eosin (H&E) to examine morphology. Figures 2(a) and 2(b) show low and high magnification images of a section of the normal mouse lung immunolabelled for caspase-1, while Figures 2(c) and 2(d) show the corresponding H&E restaining. The caspase-1 positive-staining cells around the alveoli were identified as both alveolar macrophages localised in the alveolar air space (e.g., arrowed cell in Figure 2(D1)) and type 2 alveolar cells (ATII) typically seen at alveolar junctions and showing lamellar bodies in the cytoplasm (Figures 2(D2) and 2(D3)). The identity of these two cell types was confirmed by dual labelling of caspase-1 with either F4/80 (a marker of mouse macrophages, Figure 2(e)) or SP-D (a marker of ATII, Figure 2(f)).

Thus, the normal murine airway epithelium (bronchial epithelium and ATII) expresses, at the protein level, inflammasome components Nlrp3, ASC, and caspase-1, as well as the substrate cytokines IL-1β and IL-18.

3.3. Presence of Active Caspase-1 in Inflamed, but Not Normal, Mouse Airway Epithelium. The antibody for caspase-1 used in the previous series of experiments did not differentiate between the inactive precursor and active form. Using antibodies specific for the cleaved ends of P10 and P20 subunits of caspase-1, we were able to demonstrate very low levels or absence of active caspase-1 in the control mouse lung epithelium (Figure 3(a)). In contrast, distinct patterns of active caspase-1 staining were detected in the epithelium of the inflamed airways of OVA-treated animals (Figures 3(b)–3(d)). In adjacent serial sections of inflamed lung, the two different antibodies P10 and P20 revealed the same punctate patterns of caspase-1 activation near the epithelial apical surface (Figures 3(c) and 3(d)). Preabsorption with the relevant immunogen peptides reduced the labelling of active caspase-1 in the inflamed airway epithelium to a level comparable to that of the conjugate alone (Figures 3(e)–3(g)). Because there are inherent problems in trying to quantify actual changes in fluorescence intensity where there is a redistribution of the label within the treatment accompanying the induction of inflammation, we have not attempted to quantify changes in intensity of immunofluorescence for the various proteins in these sections.

These results suggest that inflammation of the airways in mice is accompanied by conversion of zymogen caspase 1 to its active form and a more apical distribution of inflammasome proteins.

3.4. Translocation and Luminal Shedding of the IL-1 Cytokines and Inflammasome Proteins in Allergen-Induced Inflamed Airway Epithelium. As mentioned earlier, our antibody panel

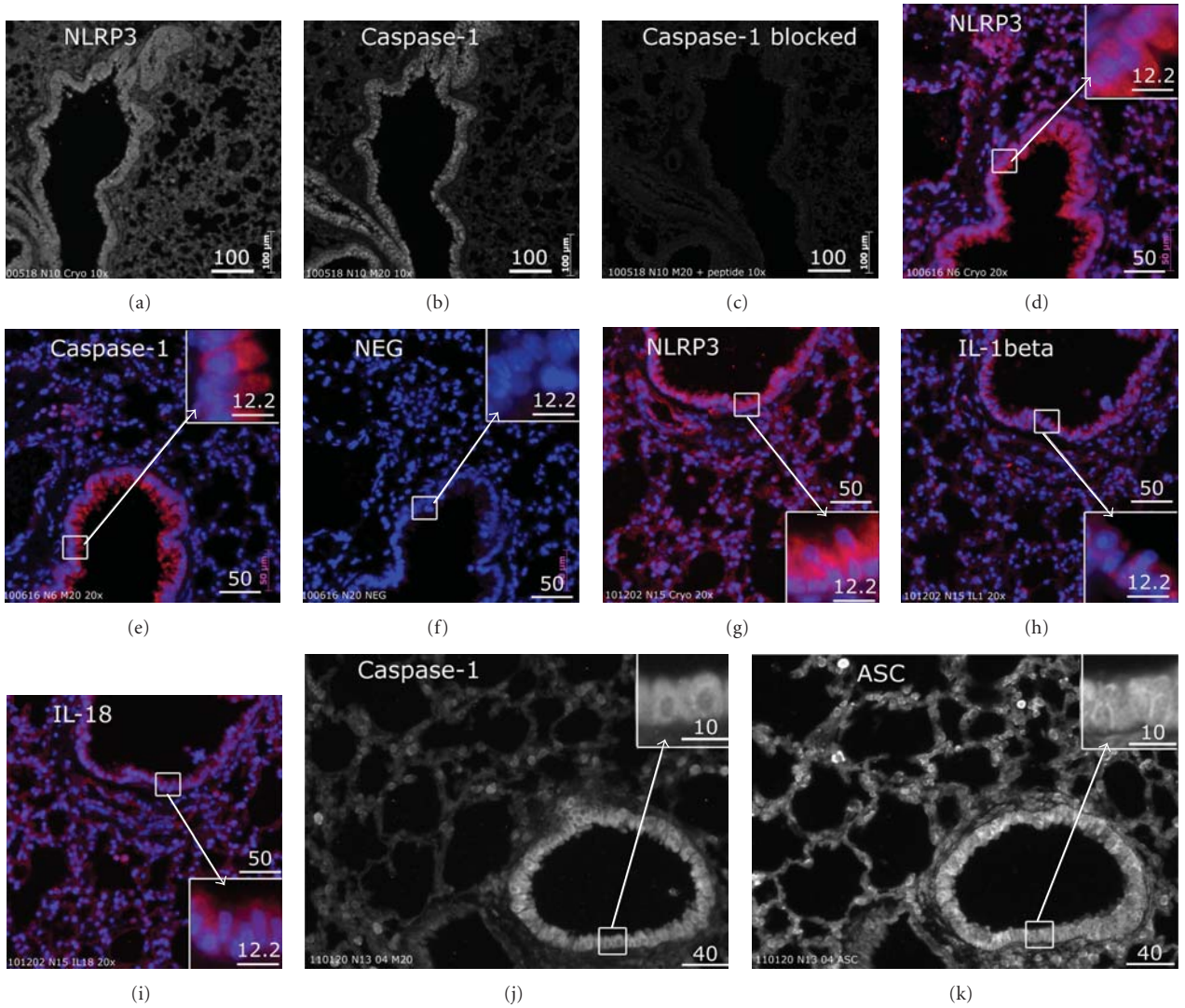


Figure 1: Expression of inflammasome proteins and the IL-1 cytokines in normal mouse lung epithelium. (a–c) Serial sections showing positive labelling for Nlrp3 (a) and total caspase-1 (b) in epithelia of three bronchioles; labelling for caspase-1 was blocked by specific peptide. No blocking peptide for Nlrp3 was available. (d–k) Serial sections showing positive and colocalized labelling of Nlrp3 and caspase-1 (d-e, red), Nlrp3, IL-1β, and IL-18 (g–i, red), and caspase-1 and ASC (j-k). (f) Negligible fluorescence was detected using normal rabbit IgG as a negative control for Nlrp3 and all other rabbit antibodies employed in this panel. Blue is DAPI counterstaining of nuclei. Images are representative of similar results from 8 control mice challenged with saline and 3 naive mice. The scale bars are in microns.

did not differentiate between the inactive precursors and the mature (active) forms of IL-1β and IL-18 cytokines. However, there were different patterns of IL-1β and IL-18 distribution demonstrated by immunofluorescence between the epithelia of OVA-treated and control mice (Figure 4). In the healthy epithelium, both cytokines distributed more or less homogeneously in the cytoplasm (Figures 4(a) and 4(b)). In contrast, in the inflamed airways, the immunofluorescence of the IL-1 cytokines had a more speckled appearance at the apical surface of the epithelium (Figures 4(c) and 4(d) arrows). Furthermore, immunofluorescence of both IL-1β and IL-18 could be detected in the lumen (Figures 4(c) and 4(d), arrowheads). Similarly, luminal staining near the apical surface was also detected for caspase-1 (Figures 4(e) and 4(k)), Nlrp3 (Figure 4(i)), and ASC (not shown). When labelled sections of inflamed mouse lungs were restained with H&E (Figures 4(g)–4(l)), part of the luminal fluorescence of the cytokines and inflammasome proteins was localized to infiltrating leukocytes (mostly eosinophils) or well preserved cell bodies (~6–10 μm in diameter) often seen embedded in the mucus near the epithelial apex (Figure 4(h) and 4(l) insets). It is unlikely that the luminal staining was an artefact caused during tissue processing. Tissues were immediately fixed in formalin for paraffin embedding and the luminal staining was consistently observed in tissue sections, including serial adjacent sections (e.g., Figures 3(c) and 3(d), Figures 4(c)–4(e), with Figure 4(f) as negative control), in all of the mice tissues analysed.

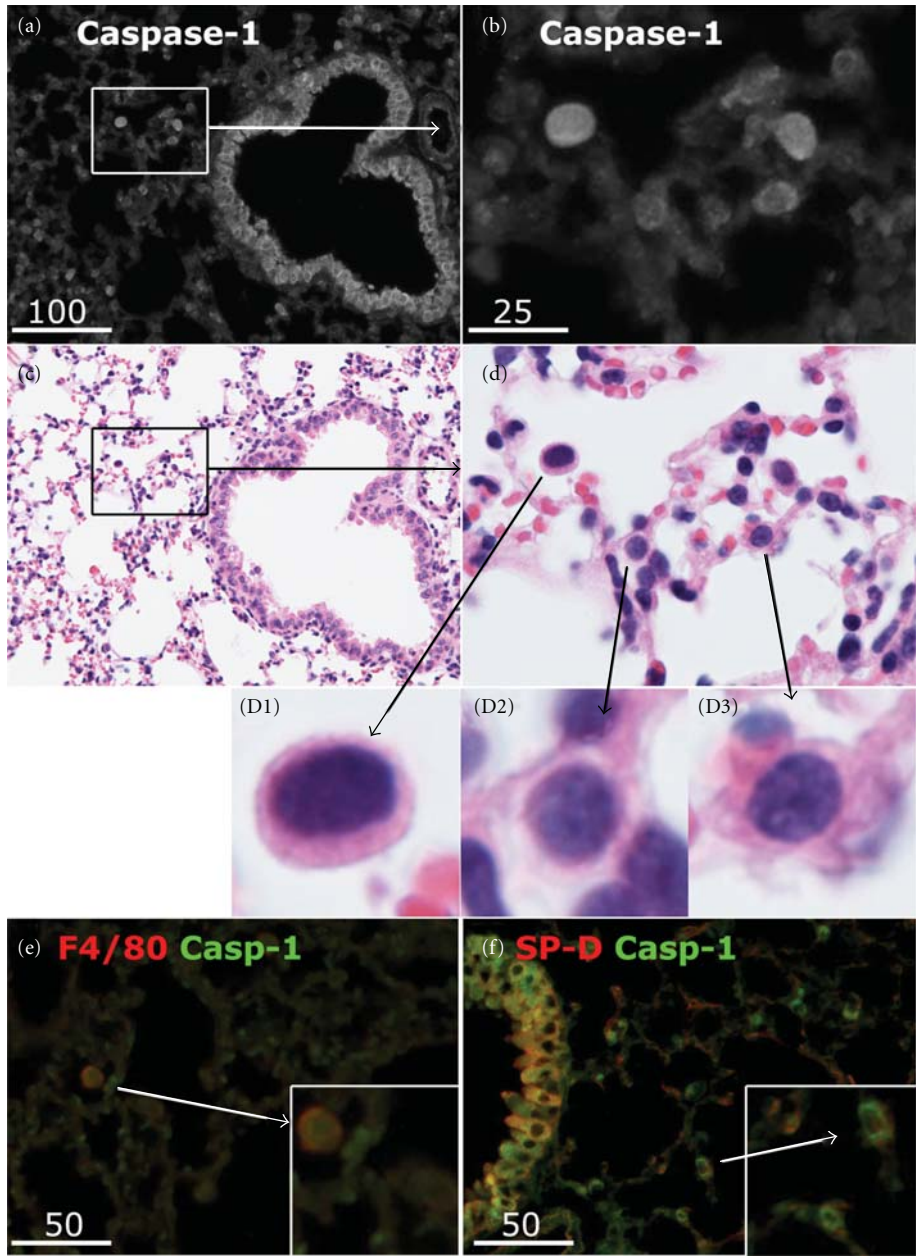


Figure 2: Identification of cell types expressing caspase-1 in normal mouse lung. (a) Representative immunofluorescence image of a normal lung section showing total caspase-1 localized to bronchiolar epithelium as the most distinct site, as well as cells scattered among alveoli (b). (c) and (d) Corresponding images of (a) and (b) following restaining with H&E. Caspase-1 positive cells included alveolar macrophages (D1) or type II alveolar cells, typically found at the alveolar interseptal junctions and containing vacuole-like lamellar bodies in the cytoplasm (D2, D3). (e) Dual labelling of a lung section for the macrophage marker F4/80 (red) and total caspase-1 (green), revealing colocalization (yellow) in macrophages (inset). (f) Dual labelling of a lung section for surfactant protein D (SPD, red) and caspase-1 (green), revealing colocalization in type II alveolar cells (inset). Scale bars are in microns.

3.5. Inflammasome Components in Other Relevant Airway Tissues of Mice with Airway Inflammation. Immunostaining of consecutive adjacent tissue sections with caspase-1 and CCR3, a marker of eosinophils, showed colocalization of CCR3 with caspase-1, detected by antibodies to either the total or the active forms of the caspase (not shown). As CCR3 is not a specific marker of eosinophils but can also be expressed in some other cell types, immunolabelled sections were restained by H&E to identify the type of infiltrating cells positively stained for inflammasome components. In accordance with our previous results >80% of these cells were eosinophils which infiltrated both the submucosal tissue and the airway lumen (Figures 4(h)–4(l)).

Another cell type which might contribute to inflammasome activation in the OVA-induced airway inflammation model is the vascular endothelium. Whereas in saline-treated

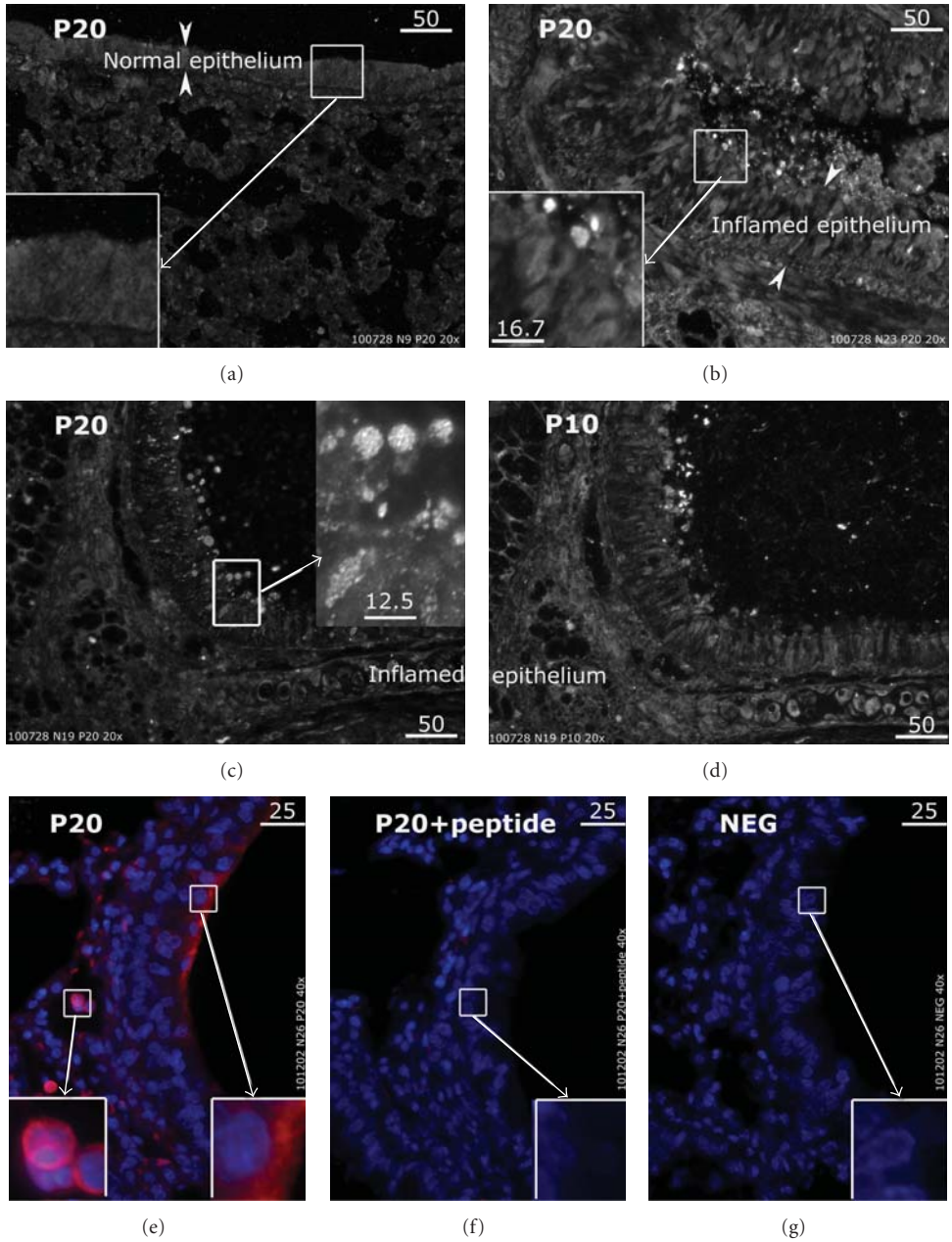


Figure 3: Activation of caspase-1 in inflamed airway epithelium of OVA-treated mice. Antibodies specific to cleaved ends of caspase-1 P20 or P10 subunits were employed to detect activation of caspase-1 in mouse lung tissue. (a) The normal airway epithelium typically expressed little active caspase-1. (b) In the inflamed airway, the swollen epithelium (arrowheads) labelled positively for active caspase-1 as shown by the punctate fluorescence of P20 near the apical surface. (c) and (d) Adjacent serial sections of an inflamed lung stained for antibodies to P20 and P10, respectively, revealed the same punctate patterns of caspase-1 activation near the apical surface. (e–g) In serial sections of an inflamed lung, fluorescence of P20 active caspase-1 antibody (e, red) was inhibited nearly completely by preabsorption of the antibody with the relevant immunogen peptide (f), to a level comparable to that of the conjugate alone control (g). Blue: DAPI counterstaining of nuclei. Images (a-b) are representative of results from P20 staining experiments on multiple sections obtained from 8 control saline-treated mice and 8 OVA-treated mice. Colocalization of P20 and P10 staining (c-d) and P20 peptide blocking experiments (e-f) were carried out on serial sections obtained from 4 inflamed lungs. Scales are in microns.

mice the vascular endothelium stained weakly for total caspase-1, it often stained strongly in the lungs of OVA-treated mice (data not shown). Unfortunately, we could not apply the antibodies to active subunits of caspase-1 to study activation of endothelial caspase-1, due to the nonspecific binding of these antibodies to red blood cells, which was not blocked by the specific peptides (data not shown). The expression of total caspase-1 protein in inflamed but not healthy endothelium was completely removed by blocking with specific peptide (data not shown).

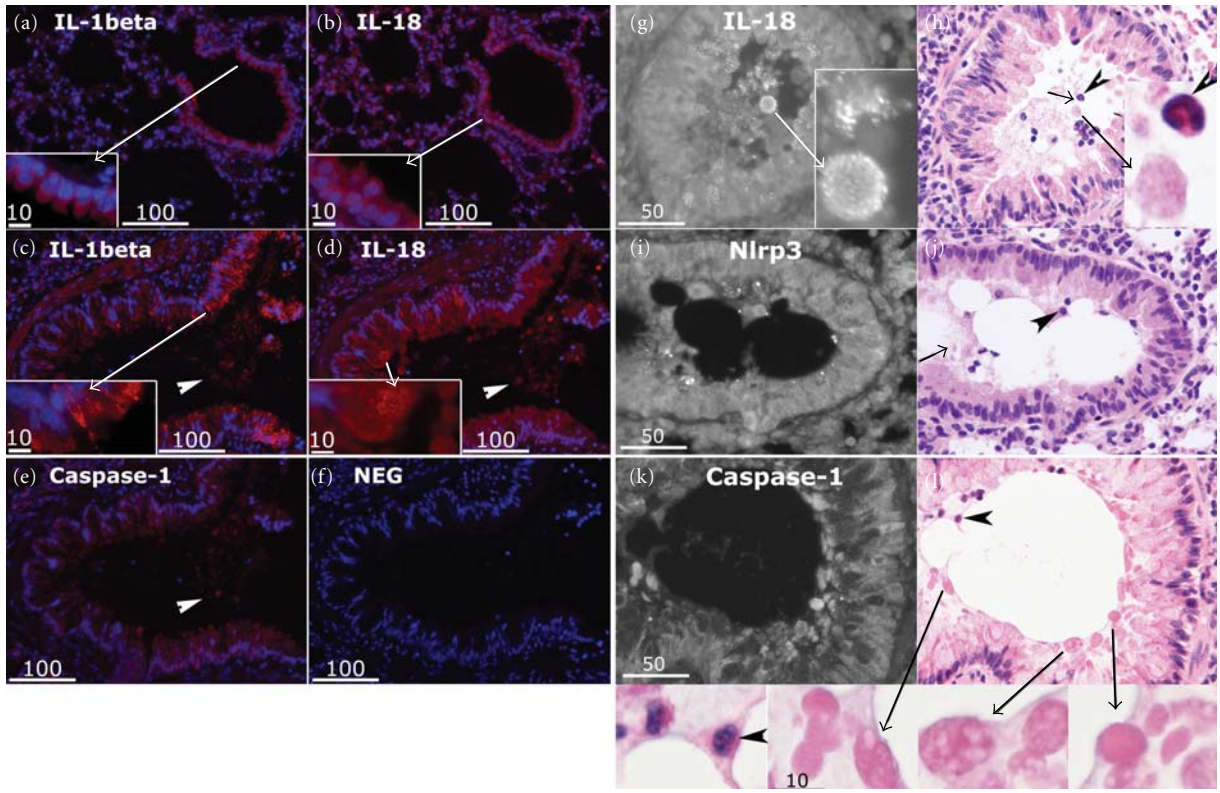


Figure 4: Translocation and luminal shedding of the IL-1 cytokines and inflammasome proteins in inflamed airway epithelium. (a–f) Serial sections of a control mouse lung (a-b) and those of an OVA-treated mouse (c–f) were stained for the cytokines and total caspase-1 in red (Cy3), and counterstained for nuclei with DAPI (blue). In the control mouse lung epithelium, IL-1β (a), IL-18 (b), and total caspase-1 (not shown) typically gave an homogenous cytoplasmic fluorescence (*insets*). In staining of multiple sections from the lung of OVA-treated mice, punctate fluorescence of both cytokines was consistently detected near the apical surface (c-d, *insets*). Together with caspase-1, these cytokines were detected in the lumen (c, d, e, *arrowheads*). (f) Control section incubated with the secondary antibody alone. Fluorescence images are representative of similar results from 3 experiments on 8 control and 8 OVA-treated mice. The scale bars are in microns. (g–i) Fluorescently labelled sections of inflamed lungs (g, i, k) were restained with H&E (h, j, l) to identify luminal structures containing inflammasome components and cytokines. Although some fluorescence was localized to infiltrating eosinophils (arrowheads) or cell-free debris (short arrows), most of the luminal shed material appeared to be within well-preserved eosinophilic vesicles (~6–10 microns) near the airway epithelial apex (inset long arrows). H&E images are representative of results from experiments carried out on multiple sections of 4 OVA-treated mouse lung. The scale bars are in microns.

3.6. Effects of Mouse Airway Inflammation on Whole Lung Gene Expression. To look at the effect of airway inflammation on expression of gene products with relevance to the inflammasome, we analysed mRNA from lungs of SAL and OVA-treated mice by superarray (Table 1). Typically, there were large increases (8–10 fold) in chemokines (Ccl12, Ccl7, and Cxcl3) known to be important in the mouse airway inflammation model. There were also significant increases with OVA-treatment in expression of a Th2 cytokine IL-6. For the IL-1-like family members, there was a 2.5-fold increase in gene expression for IL-33 and 2- and 11-fold decreases in those for IL-1β and IL-18, respectively.

Of the gene products involved in inflammasome regulation, the largest change was an 8-fold increase in the neuronal inhibitor of apoptosis protein NAIP, followed by an almost 4-fold decrease in Nlrp3 expression. Small significant increases were seen for ASC, caspase-1, and AIM2. There were no significant changes in expression of Nlrp1 and Nlrp4 (Table 1).

3.7. Cell Death and Uric Acid Levels in Airway Epithelium. In accordance with our previous data [23], using the TUNEL assay we confirmed an increased cell death in the lung sections of OVA- versus SAL-treated animals (data not shown). An analysis of BALF in the asthmatic mice demonstrated a significant increase in uric acid (Figure 5(a)), which is known as both a product of nucleic acid catabolism in dead cells and a stimulus for Nlrp3 inflammasome activation [20].

3.8. Cytokines in BALF and Serum. We were unable to detect IL-1β and IL-18 in the BALF of either saline- or OVA-treated mice (data not shown). This may be due to excessive dilution of the BALF during the lavage or choosing the wrong time-point (22 hr after final challenge) for lavaging.

Analysis of the sera showed high levels (>200 pg/mL) of IL-1β in 4 of the 8 OVA-treated mice but in none of the 8 saline-treated control mice. The serum levels of IL-1β were tightly correlated with those of TNF-α ($r = 0.95$), suggesting

Table 1: Ovalbumin-induced changes at mRNA level in mouse lung.

Gene product*	Relevance	Ratio** OVA/SAL	*P* value***
Ccl12	Chemokine of CC family which attracts eosinophils and monocytes	10.64	0.008
Ccl7	Chemokine of CC family which attracts monocytes and regulates macrophage function	8.98	0.018
NAIP 1 (neuronal apoptosis inhibitor protein)	Endogenous caspase inhibitor which binds to the inflammasome in neuronal and nonneuronal cell types	8.57	0.000
Cxcl3	CXC chemokine which controls migration and adhesion of monocytes	8.31	0.016
IL-6	Th2 cytokine which promotes airway inflammation in mice	3.08	0.050
IL-33	IL-1 like cytokine which mediates eosinophilia in mouse asthma model	2.54	0.001
ASC	Adaptor protein which recruits caspase-1 to the NLRP3 complex	2.11	0.001
Caspase 1	When activated, it processes pro-IL1b and pro-IL18 to mature cytokines	2.07	0.000
Aim2 (absent in melanoma gene 2)	Component of the AIM2 Inflammasome which activates caspase-1 independent of NLR proteins	2.00	0.002
NLRP1a	Component of the NLRP1 Inflammasome which activates caspases 1 and 5	1.07	0.658
NLRC4 (IPAF)	Component of the NLRP4 Inflammasome which mediates response to *S. typhimurium*	0.90	0.434
IL-1β	Member of IL-1 family of cytokines	0.48	0.003
NLRP3	Component of the NLRP3 Inflammasome which activates caspases 1	0.28	0.000
IL-18	Member of IL-1 family of cytokines	0.09	0.000

*Data from a superarray experiment on lung RNA from 8 SAL and 8 OVA-treated mice. Genes relevant to the asthma/inflammasome pathways are shown. **Fold up- or downregulation for OVA-treated mice relative to saline-treated mice was determined by the $\Delta\Delta$Ct method. Ratios are in decreasing order; ratios >1 represent increased expression with airway inflammation and ratios <1 represent decreased expression with airway inflammation. Upregulation of Caspase-1 and downregulation of IL-1β were also confirmed by an "in-house" RT-PCR assay. ***Student *T*-test.

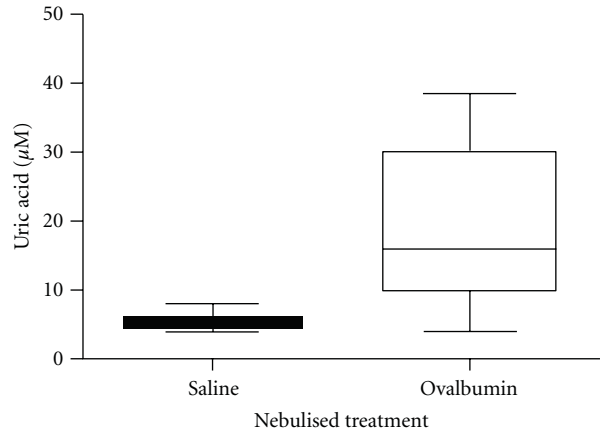
systemic effects in this model of allergic airway inflammation (Figure 5(b)).

3.9. Low Expression of Nlrp3 and IL-1β in Normal Human Bronchial Epithelial Cultures and Up-regulation by LPS but Not by IFN-γ. Commercially available passage 4 primary cultured normal human bronchial epithelial (NHBE) cells were studied for expression of the Nlrp3 and IL-1β proteins, before and after priming with LPS (5 ug/mL) or IFN-γ (50 ng/mL). Nlrp3 and IL-1β proteins were quantified in the same cell by dual-labelling with the respective antibodies (see methods) and using imageJ software to quantify fluorescence intensities. Between 26 and 51 cells (pooled from three randomly acquired images for each treatment) were analysed simultaneously for both proteins. Cells grown either in the absence of both agents or with IFN-γ alone expressed low levels of intracellular Nlrp3 and IL-1β as detected by immunofluorescence (and quantified using ImageJ software). Overnight stimulation with LPS or LPS plus IFN-γ resulted in significant increase ($P < 0.05$) of both Nlrp3 (~2 fold) and IL-1β (4-5 fold, Figure 6).

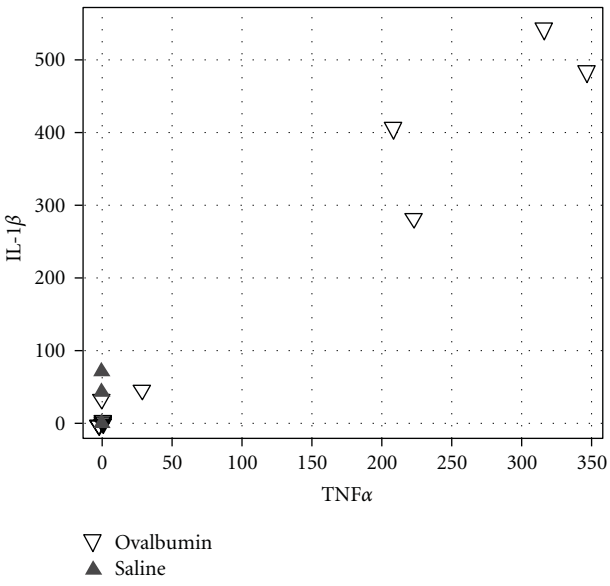
4. Discussion

This study provides the first evidence for presence of precursor components of the NRLP3 inflammasome in normal murine airway epithelium and some other resident or inflammatory airway cells. It also shows qualitative changes in the distribution of these markers, including appearance of active caspase-1, during airway inflammation in the acute mouse OVA model. Further studies are warranted to establish the functional significance of inflammasome activation as well as links between inflammasome regulation and other known mechanisms in asthma such as Th2 responses, IgE production, and its fixation on innate immune cell surfaces, eosinophilia, and airway tissue remodelling.

Our findings suggest a role for inflammasomes in innate immune responses of the airway epithelium and reinforce the hypothesis that airway epithelium plays a sentinel role in the innate immune response to inhaled microbes, allergens, and other pathogens. In particular, we have shown that the components necessary for mounting of a rapid protective inflammatory response via inflammasome activation are all



(a)



(b)

Figure 5: Increase of uric acid in BALFs (a) and cytokines IL-1β and TNF-α in sera (b) of OVA-treated mice, compared to saline-treated controls. (a) Box plot (showing median, interquartile range, and nonoutlier range) demonstrating that BALF uric acid levels are elevated in OVA-treated mice ($n = 8$) compared to SAL mice ($n = 8$), $P < 0.001$ (Kruskal-Wallis test). (b) Scatter plot of serum IL-1β versus TNF-α levels (pg/mL) in OVA-treated mice (open triangles, $n = 8$) versus SAL-treated mice (closed triangles, $n = 8$). High cytokine levels were only observed in the OVA-treated mouse group.

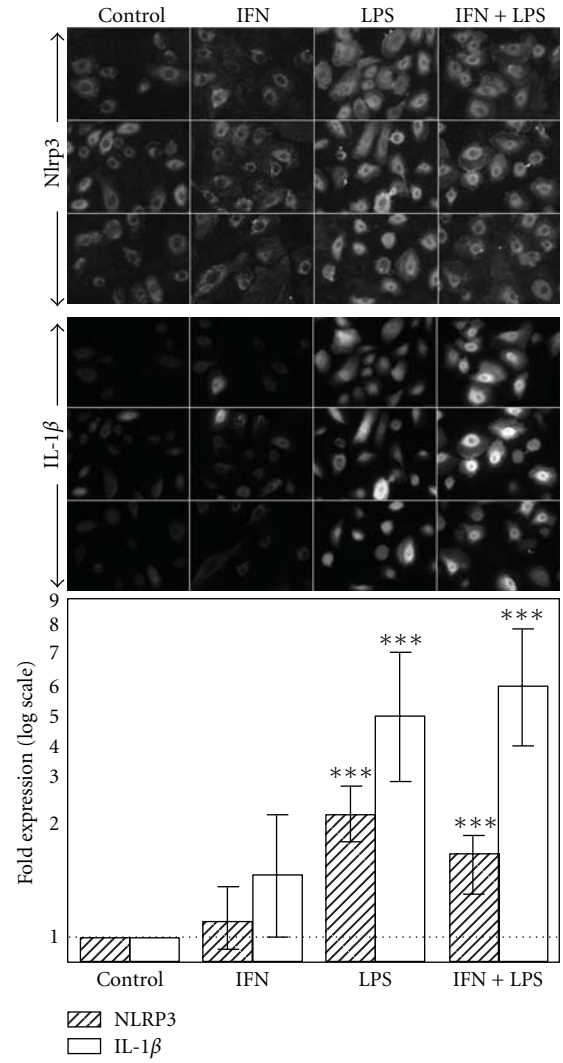


Figure 6: Induction of IL-1β and Nlrp3 protein expression in primary cell cultures of normal human bronchial epithelium by proinflammatory stimulus. Normal human bronchial epithelial (NHBE) cells were cultured without stimuli (control), or in presence of 50 ng/mL IFN-gamma (IFN), or 5 ug/mL E coli lipopolysaccharide (LPS), or their combination (IFN + LPS). Top and middle: fluorescence images of NHBE dual-labelled for intracellular Nlrp3 and IL-1β. Bottom: quantitation of fluorescence intensity by ImageJ software showing statistically significant increase ($P < 0.05$), when normalized to untreated controls, of both Nlrp3 and IL-1β in cells stimulated with LPS, or IFN + LPS combination. Error bars represent SEM. Expression of NRLP3 and IL1β was highly correlated within individual cells ($r = 0.73$, Spearman Rank correlation). Images are representative of 2 experiments with 30–50 cells analysed for each treatment.

expressed in the murine airway epithelium prior to inflammatory stimulation. Of interest, IL-1β and IL-18 were both expressed intracellularly within the normal murine airway epithelium. A similar expression of IL-1β was detected in rat lung epithelium tissue (data not shown). The failure to detect IL-1β in the circulation of the control mice suggests either that airway epithelium contributes very little to blood IL-1β levels or that signals leading to release of IL-1β (and probably also IL-18) are absent from normal airway epithelium. Our finding of intracellular IL-1β and IL-18 is therefore in keeping with the hypothesis that the airway epithelium is an important sentinel in the innate immune response and is primed for a rapid response when exposed to danger signals but has yet to undergo inflammasome activation.

Studies in cells of monocytic lineage have shown that to mount a proinflammatory response, the inflammasome pathway works in synergy with receptors for pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), the best characterized of which

are TLRs. Thus, cell lines in their normal state usually do not express precursors of IL-1β and IL-18; production, maturation, and extracellular release of these potent proinflammatory cytokines require at least two signals [7]. Ligation of TLRs leads to enhanced transcription of the IL-1β gene and accumulation of an immature form of IL-1β in the cytoplasm. Stimulation with substances such as extracellular ATP or uric acid leads to a second signal that results in caspase-1 activation, processing of pro-IL-1β, and release of the mature cytokine. Using antibodies that detect the presence of neoepitopes in the caspase-1 subunits which become accessible only upon cleavage/activation of the proenzyme and a blocking peptide, we were able to provide qualitative evidence for presence of active caspase-1 in the airway epithelium of the OVA-treated mice.

We also found, in these OVA-treated mice, a potentially interesting and novel phenomenon of subcellular translocation for epithelial IL-1β, IL-18, and caspase-1, whereby these molecules were seen to be concentrated at the epithelial apical surface and apparently shed into the airway lumen in the form of cell bodies. The significance of this is unclear but may provide a mechanism for release of inflammasome-relevant molecules into the mucosa. IL-1β and IL-18 are proteins without a signal sequence [24]. The precursors accumulate in the cytosol following translation and a second signal is required for caspase-1-mediated cleavage of the precursors to the mature form that is released from the cell. Various mechanisms for release of the active IL-1 cytokines (and caspase-1, itself) have been postulated, including preexport into secretory lysosomes or encapsulation in microvesicles or exosomes [25]. Evidence for release as cell bodies or smaller vesicles has been provided by studies in monocytes [24, 26], microglia [27], and dendritic cells [28]. The clinical relevance of the luminal shedding of the IL-1 cytokines is yet to be explored.

Our findings support the notion that the airway epithelium can itself mount a proinflammatory response via activation of its inflammasome complexes and consequent release of IL-1β and IL-18. We were able to detect high levels of IL-1β in the circulation but not in the BALF. The reason for failure to detect the IL-1-like cytokines in BALF may be a consequence of dilution of apical secretions during our lavage procedure (total wash volume was 3 mL) or may be because the time interval between final OVA challenge and BALF collection (22 hr) resulted in missing the peak cytokine response. Relevant to this, 4 of the 8 OVA-treated mice had high levels of IL-1β and TNF-α in their serum while the other 4 mice (like all of the control mice) had very low levels of both cytokines, despite all 8 OVA-treated mice having high levels of airway inflammation as evidenced by eosinophilia in BALF and tissues as well as epithelial swelling and cell death. Our immunofluorescence studies also suggested that other potential sources of IL-1-like cytokines are eosinophils, alveolar macrophages, type II alveolar cells, and endothelium.

Relevant to our finding of NRLP3 in the normal mouse bronchiolar epithelium is the recent observation reporting NRLP3 and two other NLRs (NOD1 and NOD2) in upper airway human tissues including normal nasal mucosa, nasal polyps, tonsils, and adenoids [29]. It is likely therefore that inflammasome components are present along the entire length of the airways and constitute a front-line defence. Their involvement in allergic and inflammatory diseases of the airways such as chronic rhinosinusitis, rhinitis, and asthma warrants further study.

A major limitation with studies of the whole lung is that the lung is a complex organ comprising a number of tissues. During airway inflammation, there is also substantial infiltration of inflammatory cells, especially eosinophils. It is therefore difficult to interpret changes in gene expression at the level of a single cell type in the lung. Because of this we were driven from the start at establishing the techniques to show the presence of inflammasome components in specific cell types of the lung at a histochemical level. We think this is a major advantage of our study.

Our initial attempt to look at changes in expression of inflammasome-related genes in the context of murine airway inflammation showed a large increase in expression of the caspase inhibitor and inflammasome-binding protein NAIP as well as decreases in expression of IL-18 and NRLP3. Decreases in NRLP3 and IL18 gene expression during airway inflammation may indicate the existence of a negative feedback mechanism. Since NAIP is also able to substitute for NRLP3 and other NRLs in formation of the inflammasome complex [30], there may be a switch from NRLP3 to NAIP as a result of the inflammation. Discordance between mRNA and protein expression of P_2X_7 receptor, an important upstream regulator of the NRLP3 inflammasome, has also been reported in inflamed intestinal epithelium from patients with inflammatory bowel disease [31]. In that study, in which P_2X_7 receptor engagement was required for production and release of IL-1β, mRNA levels for P_2X_7 receptor were increased while protein levels were strongly decreased. The decrease in protein levels was much greater in patients with active disease compared to those with quiescent disease and correlated with the degree of polymorphonuclear infiltration into the epithelium (transepithelial migration). The authors speculated that the decrease in protein expression may help to protect the intestinal epithelial cells from excessive activation of P_2X_7 receptor and subsequent cell death during the neutrophil transmigration. Eosinophilic infiltration into the airway epithelium is also important in the pathogenesis of asthma [32]. Downregulation of NRLP3 protein may have a similar protective role against inflammation-associated cell death during episodes of asthma.

Dissection of the mechanisms involved in airway epithelial inflammasome regulation and activation will require the use of *in vitro* airway epithelial cultures. We have shown that the presence of NRLP3 and Il-1β proteins in the commercially available normal human bronchial epithelial primary cells and the 2–5-fold upregulation of these proteins following priming with LPS indicates involvement of TLR4 in the priming. Another proinflammatory stimulus INFγ which acts via a distinct signalling pathway was ineffective.

In conclusion, we have demonstrated the presence of precursor components of the NRLP3 inflammasome in healthy murine airway epithelium as well as changes in the subcellular distribution of these components and appearance of active caspase-1 in the epithelium of inflamed airways. At

this stage we know little about the extent to which the innate immune system and inflammasome activation in particular exert protective or detrimental effects in asthma. Further studies using both lung biopsies from patients and mechanistic studies in polarized cultures of human airway epithelium are warranted and may reveal new insight into the disease mechanism and additional targets for therapeutic and/or diagnostic applications in asthma.

Abbreviations

Abs: Antibodies
ASC: Apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATII: Type 2 alveolar cells
BALF: Bronchoalveolar lavage fluid
DAMP: Danger-associated molecular pattern
HBSS: Hanks balanced salt solution
IL-1β: Interleukin-1β
IFN-γ: Interferon γ
LPS: lipopolysaccharide
NHBE: Normal human bronchial epithelial cells
NAIP: Neuronal Apoptosis Inhibitor Protein
NLR: NOD-like receptor
NOD: Nucleotide-binding oligomerization domain
OVA: Ovalbumin
PAMP: Pathogen-associated molecular pattern
PBS: phosphate-buffered saline
SAL: Saline
SP-D: Surfactant protein D
TBST: Tris-buffered saline containing Tween 20
TLRs: Toll-like receptors
TNF-α: Tumour necrosis factor-α
XIAP: X-linked inhibitor of apoptosis protein.

Disclosure

During the revision of this manuscript, an article confirming the involvement of Nlrp3 in murine airway inflammation appeared in the Press. [33] Our study differs from this one in that we immunolocalized inflammasome components to the airway epithelium.

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Review Article

Mechanisms of Remodeling in Asthmatic Airways

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Asthma is a chronic inflammatory airway disorder characterized by airway hyperresponsiveness and reversible airflow obstruction. Subgroups of asthma patients develop airflow obstruction that is irreversible or only partially reversible and experience an accelerated rate of lung function decline. The structural changes in the airways of these patients are referred to as airway remodeling. All elements of the airway wall are involved, and remodeled airway wall thickness is substantially increased compared to normal control airways. Airway remodeling is thought to contribute to the subphenotypes of irreversible airflow obstruction and airway hyperresponsiveness, and it has been associated with increased disease severity. Reversal of remodeling is therefore of paramount therapeutic importance, and mechanisms responsible for airway remodeling are feasible therapeutic targets for asthma treatment. This paper will focus on our current understanding of the mechanisms of airway remodeling in asthma and potential targets for future intervention.

1. Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by airway hyperresponsiveness (AHR) and reversible airflow obstruction that fluctuates over time. Asthma used to be considered a single disease entity, but is increasingly recognized as a disease with multiple subphenotypes that differ in clinical severity, pathological findings, response to therapy, and long-term outcome [1]. A subgroup of this heterogeneous group of asthma patients manifests airflow obstruction that is either irreversible or only partially reversible. Furthermore, some of these patients experience an accelerated rate of decline in respiratory function compared to healthy controls [2, 3]. In children with asthma defined by wheezing diagnosed at age 7 and followed for 21 years, lung function was essentially normal in patients who ceased wheezing but was increasingly abnormal in those patients who continued to wheeze frequently throughout life [4].

Airway inflammation, tissue injury, and subsequent abnormal repair lead to structural changes in the airway walls of asthmatic subjects collectively referred to as airway remodeling. Airway remodeling is strongly suspected to result in the physiologic subphenotypes of irreversible or partially reversible airflow obstruction and accelerated lung function decline [5]. Almost all elements of the airway wall have been shown to be altered in fatal asthma [6, 7]. The changes occur throughout the bronchial tree [8], but are most marked in large membranous and small cartilaginous airways [9]. Similar findings occur in nonfatal asthma, although they are less profound and localized predominantly to midsized and small membranous airways less than 3 mm in diameter [6, 10]. This review will focus on our current understanding of the pathology, pathogenesis, and physiologic consequences of airway remodeling in asthma, and discuss potential targets for therapeutic intervention.

2. Pathology and Pathogenesis of Airway Remodeling

2.1. Airway Wall Thickening. In fatal asthma, airway wall thickness is increased by between 50 and 230% compared to normal controls, while in nonfatal asthma, the increase ranges from 25–150% [11]. Increased wall thickening has repeatedly been associated with increased disease severity, including near fatal asthma [12–14]. These changes are the result of epithelial cell alterations, subepithelial fibrosis, submucosal gland hyperplasia, increased airway smooth muscle mass, and increased airway vascularization [10, 15–17].

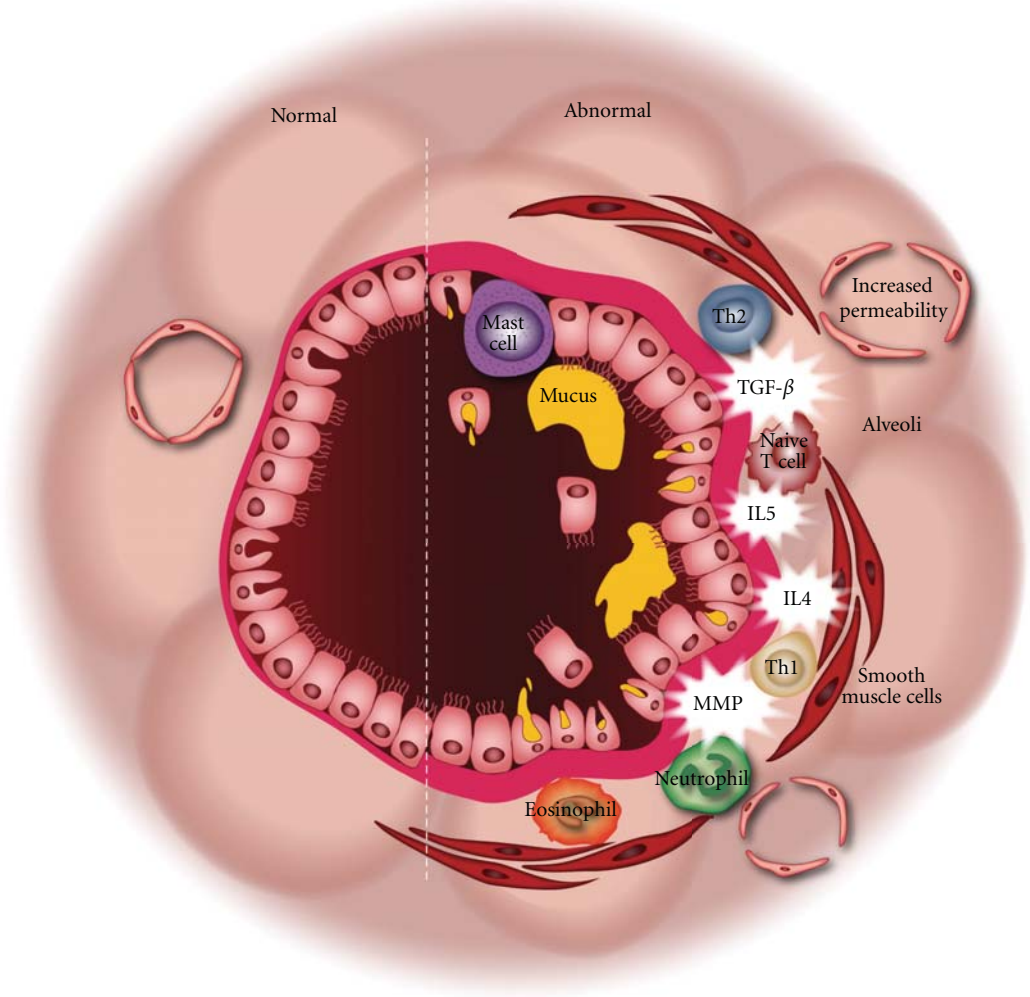

Figure 1: Airway remodeling (abnormal half of figure) involves almost all elements of the airway wall and occurs throughout the bronchial tree. Although atopy-related inflammation is considered the primary cause of asthmatic airway remodeling, insults such as tobacco smoke and viral pathogens induce a similar histologic phenotype.

Evaluation of airway wall thickening by multidetector-row computed tomography (MDCT) is a promising noninvasive technique for assessing airway remodeling [18]. Quantitative MDCT imaging allows precise measurement of airway wall area (WA) and airway wall thickness (WT) out to sixth-generation bronchi. Several studies have compared pathologic changes of airway remodeling with increased wall thickness as measured by MDCT. Both WA and WT percentages have been shown to correlate with histologic basement membrane thickening [19] and moderate correlations exist between WA and WT percentage and epithelial layer thickness [20]. In addition, quantitative CT measures, such as WA and WT, at multiple airway generations appear to correlate with FEV_1 and bronchodilator responsiveness [20]. Thus, quantitative CT scans, as a surrogate noninvasive measure of remodeling of the airways, may be used as an endpoint for targeted therapy to reverse airway remodeling or to potentially predict those individuals at risk of progressive remodeling; however, further evaluation of this modality is needed.

2.2. Allergic Airway Inflammation. Chronic inflammation that results in tissue injury with subsequent structural change during tissue repair is a well-documented biological phenomenon, for example, cirrhosis. Since chronic airway inflammation is a striking feature of asthma, asthmatic airway inflammation is often assumed to be the initiating event for airway remodeling [21]. Most, but not all, asthma is associated with atopy, and as such, asthma has largely been regarded as an allergic disease [22]. In keeping with this premise, a cellular infiltrate rich in lymphocytes, eosinophils, mast cells, neutrophils, and macrophages [5] characterizes asthmatic airway inflammation. Lymphocytic inflammation is dominated by Th2-cells producing interleukin (IL)-4, IL-5, and IL-13 (Figure 1) [23]. Overexpression of Th2 interleukins in mouse models has demonstrated changes pathognomic of asthmatic airway remodeling. Overexpression of IL-13 resulted in subepithelial fibrosis, mucus metaplasia, and an inflammatory infiltrate rich in eosinophils and macrophages [24]. IL-5 overexpression induced striking airway eosinophilia, along with mucus metaplasia and

subepithelial fibrosis [25]. While IL-4 overexpression led to eosinophilia and mucus metaplasia, subepithelial fibrosis in IL-4 overexpressing models has been unimpressive or absent depending on the study [26, 27]. In addition, mice overexpressing all three of these Th2 interleukin molecules demonstrated AHR. Eosinophils and mast cells likely impact the epithelial remodeling based on effects on barrier function, epithelial proliferation and desquamation, and goblet cell formation [28]. These data indicate that airway remodeling is quite likely driven in part by the Th2 inflammation characteristic of the asthmatic airway.

Several lines of evidence in animal models and humans suggest that the Th2 hypothesis is an incomplete explanation for asthma pathogenesis. First, many patients with asthma do not have an identifiable allergic trigger for their disease. Conversely, a significant number of patients with atopy do not develop asthma [29]. Taken together, it is reasonable to deduce that other triggers exist for generating asthma. Second, the allergic and nonallergic forms of asthma are pathologically indistinguishable from each other [30], implying that remodeling occurs independent of atopic inflammation. This suggests a remodeling pathway common to all forms of remodeled asthma. Third, current anti-inflammatory and allergen-reduction therapies (see below) do not prevent the development of asthma or effectively reverse airway remodeling once it has occurred [31]. Lastly, airway remodeling occurs simultaneously with inflammation and may indeed be necessary for the establishment of a chronic inflammatory state [32]. These observations have encouraged exploration of alternative mechanisms of airway injury as the underlying mechanism of airway remodeling.

2.3. Epithelial-Driven Models of Airway Remodeling. While it is likely that some aspects of airway remodeling are the end result of allergen exposure and subsequent chronic allergic inflammation, it is increasingly believed that predisposition to asthma lies in a structurally and functionally defective airway epithelium which links the inhaled environment to underlying airway structures. This phenomenon is best explained by the model of the epithelial mesenchymal trophic unit or EMTU proposed by Holgate [[33] and reviewed in [34, 35]]. In this model, both airway inflammation and remodeling are the consequence of repetitive environmental injury to a defective airway epithelium by viruses, air pollutants, or tobacco smoke (Figure 1) [22]. Injury leads to interaction between the dysfunctional epithelium and the underlying mesenchyme that results in amplification of the inflammatory and remodeling responses in the underlying layers of the airway wall with subsequently defective airway repair [35].

Support for this model is increasingly robust. Evidence, primarily from animal models, indicates that innate immune responses to respiratory virus infections, for example, contribute to the development of inflammatory airway disease characteristic of asthma [36–39]. Paramyxoviral infection of mice has been shown to produce acute bronchiolitis resulting in airway inflammation and AHR. However, infection also results in a chronic inflammatory response with airway remodeling and AHR phenotypes [40]. This chronic response is not only strikingly similar to the inflammatory response in the airways of asthmatic patients, but also persists for over a year after mice have cleared the virus from the airways. It has subsequently been shown that the chronic inflammatory state is related to the severity of infection and is produced by an innate epithelial immune response in which natural killer T cells activate macrophages to produce proinflammatory cytokines like IL-13, which contribute to chronic mucous cell metaplasia [[41] and are reviewed in [42, 43]]. The mouse model correlates well with clinical findings. Paramyxoviral infections are a primary cause of lower respiratory tract infection in infants and children [44], and children with severe RSV bronchiolitis are predisposed to development of a chronic wheezing illness in the absence of both atopy and viral persistence in airway tissue [45, 46].

2.4. Epithelial Cell Alterations. Epithelial cell shedding, ciliated cell loss, and goblet cell hyperplasia have all been described in asthmatic airways [6, 15, 47]. Epithelial shedding has been noted in postmortem studies of asthmatic airways, and sputum and BAL samples from asthmatic patients contain increased amounts of epithelial cells [6, 48]. However, epithelial cell desquamation in bronchial biopsy specimens from healthy nonasthmatic subjects appears similar to that seen in biopsies from mild to severe asthmatics [49, 50] suggesting that this phenomenon is related to the sampling technique itself. Evidence of increased epithelial cell proliferation contributing to thickening of the epithelium and an increased lamina reticularis (also known as subepithelial fibrosis, see below) has been observed in patients with moderate to severe asthma (Figure 2) while being absent in patients with mild persistent asthma, chronic bronchitis, and normal controls [49]. These studies suggest that thickening of the airway seen in severe asthma may be due, in part, to airway epithelial proliferation (Figure 3), although conflicting data exist (see below).

Goblet cell hyperplasia has been consistently demonstrated in mild, moderate, and severe forms of asthma (Figure 3), although the finding is particularly apparently increased in severe and fatal asthma [51, 52]. Similarly, an increase in the area of airway wall occupied by submucosal mucus glands is a frequent finding in asthmatic airways, and occurs in both fatal and nonfatal forms of asthma [6]. Goblet cells produce mucin glycoproteins (MUC), 13 of which have been identified in human airways [53]. The dominant mucin in humans is MUC5AC, which is expressed in the airways of normal subjects and is upregulated in asthmatic subjects [54]. Goblet cell hyperplasia has been demonstrated following adoptive transfer of Th2 cells into ovalbumin-challenged mice [55]. This is most likely the result of Th2-driven interleukin expression (see above). IL-13, in particular, signals through the STAT-6 signaling pathway [5] and the effects of IL-13 overexpression in mice are almost completely STAT-6 dependent [56].

Other changes observed in the airway epithelium lend support to the EMTU hypothesis of asthma pathogenesis. Epithelial injury is normally followed by upregulation of proteins responsible for tissue repair. Expression of epithelial growth factor receptor (EGFR) and MUC5AC are both

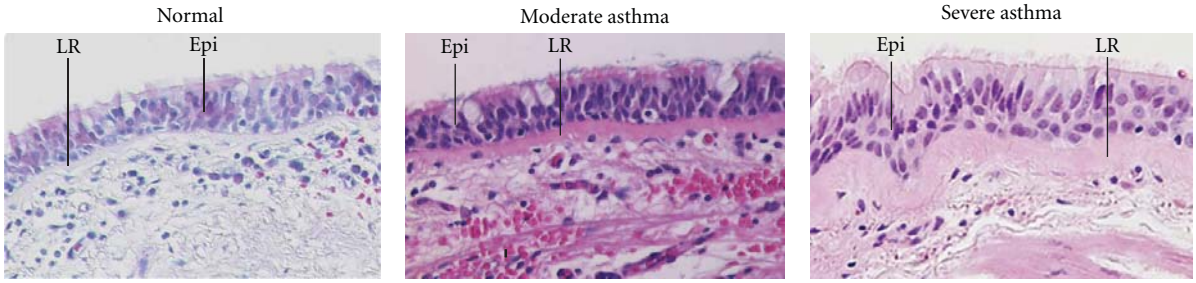

Figure 2: Hematoxylin and eosin stained endobronchial biopsies from control, moderate asthmatic, and severe asthmatic patients. Lamina reticularis (LR) and epithelium (Epi) are labeled. Note the increased thickness of both the LR and epithelium as asthma severity increases. Mag = 20x.

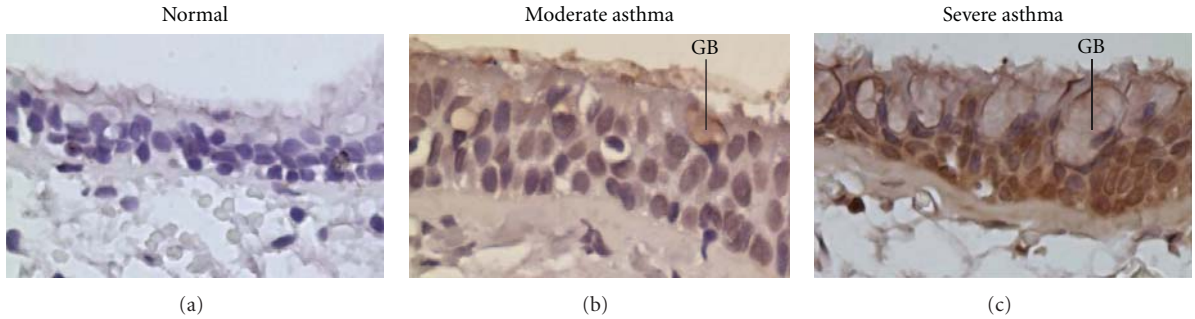

Figure 3: Endobronchial biopsy specimens from control, moderate asthmatic, and severe asthmatic patients stained with antiretinoblastoma (anti-Rb) antibody, a marker of cell proliferation. Rb-positive cells stain brown. There is a significant increase in Rb-positive epithelial cells as asthma severity increases. There is also an increase in the number and size of goblet cells (GB) in the epithelial layer as asthma severity increases. Mag = 40x.

markedly upregulated in the epithelium of asthmatic patients [57, 58], and have been shown to colocalize in goblet cells [59]. Immunoreactivity to EGFR and the total area of MUC5AC staining show a positive correlation in both asthmatics and control subjects. Furthermore, activation of EGFR has been shown to upregulate both mucin production and goblet cell generation in human epithelial cells *in vitro* [57]. Interestingly, increased airway expression levels of EGFR are not associated with markers of increased cell proliferation as would be expected in tissue undergoing active repair [60] suggesting an innate defect of the asthmatic epithelium to repair itself.

2.5. Subepithelial Fibrosis. The original report of airway remodeling described the phenomenon of basement membrane thickening [61]. Electron microscopy has subsequently shown that thickening occurs just below the true basement membrane in a zone known as the lamina reticularis [17]. The lamina reticularis (Figure 2) is a collagenous layer 4-5 μm thick in control subjects. In asthmatics, lamina reticularis thickness has been documented at between 7 and 23 μm [62]. Thickening is the result of extracellular matrix deposition, primarily collagens I, III, and V [5]. In addition, abnormalities of noncollagenous matrix, including elastin, fibronectin, tenascin, lumican, and proteoglycans, have also been described [17, 63, 64].

Subepithelial fibrosis occurs in children, and is similar in extent to that seen in adults [65] suggesting that it is an early finding of asthmatic airway remodeling. Subepithelial fibrosis has also been reported in all severities of asthma [9, 66]. However, the specificity of subepithelial fibrosis is called into question by studies that have identified severe asthmatics without subepithelial fibrosis, and nonasthmatic subjects with significant fibrosis [67–69]. Furthermore several functional measurements of asthma show variable correlations with the degree of fibrosis [66, 70–72] raising questions about its functional consequences.

Myofibroblasts are probably key effectors of subepithelial fibrosis. Myofibroblasts are specialized cells with phenotypic characteristics of both fibroblasts and myocytes [53]. They express α-smooth muscle actin, produce inflammatory mediators, and are major producers of extracellular matrix proteins necessary for tissue repair and remodeling.

Transforming growth factor- (TGF-) β mediates the effects of IL-13 overexpressing mice [73]. TGF-β is a cytokine produced by multiple lung cells including epithelial cells, macrophages, fibroblasts, lymphocytes, and eosinophils [53]. TGF-β induces fibroblasts to express α-smooth muscle actin and assume a myofibroblast phenotype [74]. As part of normal wound repair, TGF-β induces expression and secretion of multiple extracellular matrix proteins while also inhibiting their degradation. In many diseases, excessive TGF-β

results in an excess of pathologic tissue fibrosis leading to compromised organ function [75]. Compared to controls, TGF-β expression is increased in asthmatic airways and BAL fluid. In addition, TGF-β levels correlate with the extent of subepithelial fibrosis, airway fibroblast numbers, and disease severity [76–78]. Thus, excess TGF-β production may be pivotal for the development of subepithelial fibrosis.

Matrix metalloproteinases are zinc-dependent endopeptidases capable of degrading extracellular matrix molecules. The dynamic equilibrium between matrix metalloproteinases and their inhibitors is a critical determinant of matrix remodeling [79]. The existence of increased subepithelial fibrosis in asthmatic airways strongly suggests that a profibrotic balance exists between the two. In asthma, the most important metalloproteinase molecules are MMP-9 and its inhibitor, tissue inhibitor of metalloproteinase- (TIMP-) 1 [5]. Both MMP-9 and TIMP-1 levels are elevated in airway biopsies and BAL fluid of asthmatic patients [80–82]. However, compared to control subjects, asthmatics have a significantly lower MMP-9 to TIMP-1 ratio supporting a profibrotic balance (inhibition over degradation). In addition, the lower MMP-9 to TIMP-1 ratios correlate with the degree of airway obstruction [83].

TGF-β is secreted from cells as a latent complex and is targeted to the extracellular matrix by latent TGF-β binding proteins for subsequent activation [84]. MMPs regulate matrix-bound cytokine release [83], and activation of TGF-β is MMP-9 dependent [73]. Therefore, the role of elevated levels of MMP-9 in asthma may be related to TGF-β activation and its downstream fibrotic sequelae [5].

Thickening of the lamina reticularis provides further evidence for the idea of a dynamically interactive EMTU. Epithelial disturbances have been shown to result in increased levels of fibrogenic growth factors including both the latent and active forms of TGF-β [33, 85]. This response is enhanced in asthmatic epithelium compared to normal controls, and the fibrogenic factors have been shown to localize in mesenchymal elements underlying the injured epithelium [86, 87] including the lamina reticularis. Thus, the lamina reticularis may act as a conduit for transmission of signals from an innately defective epithelium to deeper tissues of the airway wall.

2.6. Increased Airway Smooth Muscle Mass. Increased airway smooth muscle (ASM) mass is the most prominent feature of airway remodeling [6], with ASM mass increasing disproportionately compared to the increase in total wall thickness [53]. It has been documented in both fatal and nonfatal asthma [11], and correlates with both disease severity and duration, being greater in fatal than nonfatal cases of asthma [6, 7, 10] and greater in older patients with fatal asthma than younger patients with fatal disease.

The increase in ASM mass may be the coordinated result of increased myocyte size (hypertrophy), increased myocyte number (hyperplasia), and potentially differentiation and migration of mesenchymal cells to ASM bundles [88–91]. Controversy exists regarding the relative contributions of hypertrophy and hyperplasia to ASM mass increases. The evidence for hyperplasia is relatively convincing [90, 92]. However, support for hypertrophy is conflicting, in part because documentation of increased cell size (width) may be subject to artifact resulting from cell shortening [32]. While studies have documented hypertrophy of ASM in severe asthma, particularly in smaller airways, other studies found no evidence for ASM hypertrophy in mild-moderate asthma [32].

Mitogens are chemical compounds that stimulate cell division and trigger mitosis. Mitogens play an integral role in the development of increased ASM mass typical of asthmatic airways. Mitogens bind receptor tyrosine kinases (RTK), G protein-coupled receptors (GPCR), and cytokine receptors. These receptor systems are all capable of producing increases in ASM mass in cell culture models [53]. The list of mitogens is extensive, and includes TGF-β, IL-1β, IL-6, thromboxanes, leukotrienes, histamine, tryptase, serotonin, vascular endothelial growth factor (VEGF), and numerous others [89, 93, 94]. The receptor systems regulate mitogenesis primarily through the phosphoinositide 3′-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways [95, 96]. The PI3K and ERK pathways activate transcription factors which phosphorylate D-type cyclins facilitating cell cycle progression [53]. Almost all of these mitogens have been identified in airway biopsies and BAL fluid from asthmatic patients or are detected in asthmatic airway cell cultures [21]. They may therefore represent targets for modulation of airway smooth muscle in asthmatic disease.

ASM cells are often noted in close proximity to the airway epithelium. This epithelial-muscle distance was measured at 67 μm in asthmatics compared to 135 μm in controls [70]. It has been postulated that mesenchymal airway cells differentiate into ASM with subsequent migration of the new ASM cells into muscle bundles [97]. Whether these phenomena occur *in vivo* is unknown, but reports indicate that cultured human ASM cells migrate in response to mitogenic stimuli [98]. Many of the mitogens involved in cell proliferation also induce ASM cell migration including TGF-β, IL-1β, and VEGF [21, 53].

2.7. Bronchial Neovascularization. Increased vascularity is frequently associated with chronic inflammation, and increased airway vascularity is well documented in asthma [16, 99]. Compared to controls, bronchial biopsies from asthmatic patients demonstrate an increase in the number and cross-sectional area of blood vessels, predominantly capillaries and venules, especially in the lamina propria [98, 100, 101]. It has been suggested that neovascularization worsens disease through increased vascular congestion, airway edema and inflammation, and global wall thickening [5]. In support of this idea, increases in airway vessel number have been shown to correlate with both disease severity and AHR [100, 102, 103].

VEGF is an angiogenic growth factor. It is a mitogen for vascular endothelial cells inducing endothelial cell proliferation and migration while inhibiting apoptosis. VEGF is an important factor in diseases associated with abnormal angiogenesis and wound repair [104]. Overexpression of VEGF in mice induces marked airway angiogenesis along with significant airway edema [105]. Interestingly, VEGF overexpressing

mice also demonstrate increased Th2 cytokine expression, including IL-13 and TGF-β, and have evidence of subepithelial fibrosis and increased ASM mass. Furthermore, IL-13 overexpression is associated with increased levels of VEGF suggesting that a positive feedback loop promoting Th2 polarization may exist in asthmatic airways [106]. VEGF levels in sputum and BAL fluid from asthmatics are significantly increased and appear to correlate with disease activity [98, 107, 108].

3. Physiologic Consequences of Airway Remodeling

Functional data increasingly support the idea that remodeling-induced structural changes contribute to the subphenotypes of AHR and irreversible or partially reversible airflow obstruction [109, 110]. There are many potential etiologic mechanisms that link altered airway anatomy and asthmatic pathophysiology, but most are beyond the scope of this review.

The role of small airways in producing airflow obstruction and AHR seems to be greater than that of larger airways [111, 112]. Peripheral lung resistance is increased in all severities of asthma, even mild cases [113, 114]. Heterogeneity of peripheral bronchoconstriction is a major determinant of airflow obstruction, creating large increases in the load against which patients must breath [115, 116]. Although heterogeneous bronchoconstriction is well documented in wild-type animals and nonasthmatic human subjects [117–119], mathematical models indicate that heterogeneity of peripheral bronchoconstriction is significantly increased in asthmatic patients when compared to normal controls [120, 121].

Increased ASM mass is thought to be the most likely cause of AHR [122]. Asthmatic ASM exhibits increased contractility [123, 124], a finding initially ascribed to increased total ASM mass [7, 8, 10]. However, results from studies comparing ASM force generation in asthmatic ASM and normal controls are contradictory [125–127], in large part because of the difficulty in normalizing measured force to ASM mass [128]. A more likely explanation of AHR in asthmatic patients is an increase in the maximal velocity of shortening (V_{max}) of ASM cells [128, 129]. Experimental evidence supporting an increase in V_{max} includes data from human [124, 130] and animal models [131].

Noncontractile elements of airway remodeling also contribute to AHR [109]. Increased airway extracellular matrix is one noncontractile element that may contribute to AHR. Decreased airway compliance is well documented in asthmatic patients [132, 133] and has been found to correlate inversely, although weakly, with increases in subepithelial fibrosis [134]. Therefore, increased extracellular matrix content may contribute to nonreversible airway obstruction by reducing airway distensibility. It has also been postulated that increases in extracellular matrix lead to an excess of matrix-bound cytokines and retention of soluble inflammatory mediators [135]. This results in worsening airway inflammation with subsequent chronic persistence of established AHR.

Finally, contractile and noncontractile elements of the remodeled airway wall may interact to increase airflow obstruction and AHR. Application of cyclic stresses to airway segments reduces ASM contractility [136, 137]. Decreased airway distensibility may reduce the cyclic stresses transmitted to ASM during breathing, reducing the cyclical stretching of ASM [138, 139]. ASM adapts to this attenuated stimulus by assuming a shorter resting length while retaining its ability to generate force. This mechanical plasticity, as the phenomenon is known, is an important feature of ASM biology affecting its contractile function [140]. The shortened muscle fibers enhance the airways predisposition to undergo excessive constriction during stimulation [110].

4. Therapeutic Targets for Airway Remodeling

The natural history of airway remodeling is poorly understood [9]. While the physiologic subphenotypes are more obvious in older patients with more severe disease of longer duration [141], airway remodeling is known to occur early on in the disease course [32]. Clinical trials of therapeutic intervention to prevent airway remodeling are currently lacking. Reversal of existing remodeling is therefore an important therapeutic objective since remodeling may often be present even at the time of asthma diagnosis.

4.1. "Anti-Inflammatory" Therapy. Animal studies of allergen-challenged models suggest that airway remodeling can be prevented, but also suggest that it cannot be fully reversed once initiated [142]. In general, therapies aimed at immunomodulation have proved disappointing. These include therapies directed against T cells (azathioprine, cyclosporine, and methotrexate) and Th-2 cytokine blockade (IL-4, IL-5 (see below) and IL-13) [143]. Some positive clinical data has been obtained, specifically with the use of corticosteroids, but in general, the data are mixed. In one study, treatment of asthmatic patients with inhaled corticosteroids (ICS) for 1 year demonstrated reductions in both subepithelial fibrosis and AHR [144]. The authors attributed the decrease in AHR to both reduced airway remodeling and decreased airway inflammation. However, other studies have shown mixed results. While they have demonstrated significant reductions in AHR, these same studies demonstrated either lack of airway remodeling after 8 weeks of ICS therapy [145], or no change in lung function (specifically postbronchodilator FEV_1% predicted) when compared to placebo, despite treatment with ICS for 4 to 6 years [146].

4.2. Targeted Immunotherapy. Airway eosinophils are a key component of Th2 inflammation and are thought to be key effectors of both chronic inflammation and airway remodeling in asthma [21]. IL-5 is a key mediator of eosinophil activation and results in increase in the number of circulating, airway, and sputum eosinophils in mice [147]. Initial trials with mepolizumab, an anti-IL-5 monoclonal antibody, were disappointing. Although circulating eosinophils were dramatically reduced, airway eosinophils were only depleted

by approximately 55% and there was no observable effect on circulating T cells [148, 149]. In addition, no significant effect on asthma outcomes or AHR were noted [149–151]. Subsequent trials of mepolizumab in severe asthma patients with persistent sputum eosinophilia have demonstrated a significant reduction in airway wall thickness measured by quantitative CT scanning (RB1 WA%/body surface area). In addition, significant reductions in the levels of circulating and sputum eosinophils were also noted when compared to placebo [152, 153]. However, no effects on AHR were identified.

Treatment with omalizumab, an anti-human IgE monoclonal antibody, has well-documented efficacy in improving asthma outcomes in a subgroup of patients with moderate to severe persistent asthma [154–159]. Patients in this subgroup demonstrate a confirmed atopic component and remain uncontrolled despite high-dose inhaled corticosteroids and at least one additional controller therapy [160]. Omalizumab inhibits the binding of IgE to the high-affinity IgE receptor (FcεRI). It does so by binding to an epitope on the IgE molecule to which the FcεRI would bind [161]. Omalizumab resulted in significant reductions in sputum eosinophil counts and an 80% reduction in the number of airway tissue eosinophils. Even more striking was an almost complete reduction in airway cells staining positive for FcεRI (including basophils and mast cells) and a significant decrease in the number of airway T and B cells [162].

The therapeutic utility of anti-IgE therapy stands in stark juxtaposition to that of anti-IL-5 therapy. The differences suggest that either the (relatively) small reduction in airway eosinophilia mediated by IL-5 blockade is insufficient to produce a therapeutic effect, or that the effects of anti-IgE therapy result from attenuation of multiple effector cells in the asthmatic inflammatory cascade and not solely a reduction in tissue eosinophils [35].

4.3. Bronchial Thermoplasty. Bronchial thermoplasty (BT) delivers thermal energy to the airway wall in a controlled manner to reduce excessive ASM [163]. The procedure has been well studied in severe persistent asthma that is not well controlled with inhaled corticosteroids and long-acting beta-agonists [164, 165]. Long-term followup of BT study patients supports the efficacy and safety of BT out to 5 years [166]. Lung function (FEV_1 and FVC) remained stable over five years of followup. However, while studies have established small but significant improvements in PC_{20} doubling in patients undergoing BT when compared to controls for periods of up to 3 years after the procedure [166], there has in general been a lack of evidence demonstrating reduction in AHR. In the largest trial of bronchial thermoplasty to date [164], a subset of participants (100 treated with thermoplasty, 50 received sham bronchoscopy) underwent CT scans before and one year after treatment. Qualitative analysis of these images demonstrated no evidence of airway or parenchymal injury related to bronchial thermoplasty and an increase in bronchial wall thickening in those receiving sham bronchoscopy [167]. Therefore, thermoplasty may represent a mechanism by which smooth muscle can be abrogated resulting in the prevention of progressive remodeling in severe asthma.

5. Conclusions

There is now a substantial body of evidence documenting typical structural changes in the airways and lung parenchyma of asthmatic patients. These changes most likely contribute to the AHR and irreversible or partially reversible airflow obstruction seen in subgroups of asthmatic patients, especially those with more severe disease. The mechanisms responsible for these changes present viable therapeutic targets for the prevention and treatment of airway remodeling in asthma.

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Research Article

Evaluation of Differentiated Human Bronchial Epithelial Cell Culture Systems for Asthma Research

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The aim of the current study was to evaluate primary (human bronchial epithelial cells, HBEC) and non-primary (Calu-3, BEAS-2B, BEAS-2B R1) bronchial epithelial cell culture systems as air-liquid interface- (ALI-) differentiated models for asthma research. Ability to differentiate into goblet (MUC5AC+) and ciliated (β-Tubulin IV+) cells was evaluated by confocal imaging and qPCR. Expression of tight junction/adhesion proteins (ZO-1, E-Cadherin) and development of transepithelial electrical resistance (TEER) were assessed. Primary cells showed localised MUC5AC, β-Tubulin IV, ZO-1, and E-Cadherin and developed TEER with, however, a large degree of inter- and intradonor variation. Calu-3 cells developed a more reproducible TEER and a phenotype similar to primary cells although with diffuse β-Tubulin IV staining. BEAS-2B cells did not differentiate or develop tight junctions. These data highlight the challenges in working with primary cell models and the need for careful characterisation and selection of systems to answer specific research questions.

1. Introduction

Asthma is a chronic respiratory condition characterised by recurrent exacerbations [1]. A feature of asthma (especially severe asthma) is airway remodelling, that is, increased smooth muscle mass, fibrosis, and excessive mucus production [2]. The epithelium plays a key role in the development of airway remodelling and inflammation as it represents the primary barrier to environmental exposures and also signals to other cell types within the context of the epithelial mesenchymal trophic unit [3, 4].

In vitro models using primary cells and cell lines are essential for understanding the function of the epithelium relevant to asthma. Cells are routinely cultured in submerged monolayers on a plastic substrate. In order to obtain a more physiological model, primary human bronchial epithelial cells (HBECs) may be cultured at air-liquid interface (ALI) using defined medium to drive a differentiated phenotype [5]. This model shows a pseudostratified, polarised phenotype, including ciliated and goblet cells and develops high transepithelial electrical resistance (TEER) [6, 7]. Measurement of TEER provides an indirect measure of formation of tight junctions and is often used as a marker of disruption of the epithelial layer [8].

Cultured primary HBECs from asthma and non-asthma subjects have been compared in a number of studies, to investigate intrinsic differences in the asthmatic epithelium. Epithelial cells from asthmatic patients display differential expression of genes associated with inflammation, repair, and remodelling and have been shown to differ from normal cells in culture, including increased proliferation [9] and slower repair of a mechanical wound [10, 11]. Several groups have cultured asthmatic epithelial cells at ALI, showing a less differentiated phenotype, that is, increased numbers of basal cells [12] or decreased tight junction formation [13], and differing responses to stimulation including viral infection, mechanical wounding, and cigarette smoke [12–14]. There has been some debate regarding reported differences between normal and asthmatic cells. For instance, Hackett et al. [12] report no difference in TEER between normal and asthmatic

cultures, whilst Xiao and colleagues suggest that cells from asthmatic subjects show decreased TEER and disrupted tight junctions [13]. These discrepancies may reflect differences in donor profile (donors were significantly older in the Xiao study), cell source (*post mortem* donor lungs *versus* bronchial brushings), or the much greater number of subjects included in the Xiao study. Paediatric asthmatic HBECs in monolayer culture show slower repair of a mechanical wound [10, 11]. At ALI, HBECs from asthma donors show increased cytokine release in response to mechanical wounding, or viral or particulate matter exposure [12], and are more sensitive to disruption of TEER by cigarette smoke extract [13]. Another study found that whilst HBECs from normal donors showed an increased rate of wound repair in response to IL-1β treatment, asthmatic cells did not show this response [14]. These results may suggest that asthmatic cells at ALI have an intrinsically different phenotype and show different signalling responses to normal cells and support the utility of epithelial cell culture in asthma research.

Direct comparisons of normal and asthmatic cells allow characterisation of the asthmatic phenotype; however, they are less helpful when trying to dissect the underlying mechanisms behind epithelial changes in asthma. Normal primary bronchial epithelial cells and cell lines may be used to model various aspects of asthma. Cytokines may be added to cells in monolayer or ALI culture [15–17], whilst asthma triggers such as Derp1 or rhinovirus have been applied to the cells to mimic allergen inhalation or viral exacerbation [18, 19]. Danahay et al. treated ALI HBECs with IL-13 or IL-4, resulting in changes in permeability, suggesting that these asthma-related cytokines may contribute to a more secretory phenotype [15], whilst Wadsworth and colleagues found that addition of IL-13 and other T_H2 cytokines led to increased MMP7 and FasL release, which may lead to epithelial damage and inflammation [16]. In another study, HBEC or BEAS-2B cells at ALI were treated with leukotriene D_4, resulting in signalling via EGFR and release of IL-8 [17]. Overall these data demonstrate that the use of HBEC and cell line cultures can provide a unique insight into mechanisms underlying asthma and it is important to understand the strengths and weaknesses of these culture systems.

Accumulating data suggest that bronchial epithelial cells may be a viable drug target in asthma [20]. Cell culture models are used in drug development, both to assess the direct effect of potential drugs on cell function and signalling and to investigate drug uptake and metabolism [21]. Although primary cells are the gold standard, there are some disadvantages to their use including cost, limited life span, and variability between donors, passage, or experiments. Primary cells may also be more difficult to transfect or otherwise manipulate. This has led to the use of cell line systems, in both monolayer culture and at ALI. The Calu-3 cell line was established from a pleural effusion of a lung adenocarcinoma, derived from submucosal gland serous cells [22–24]. It is often used at ALI as a model system, particularly for investigations of tight junction and barrier formation [23], for instance, showing that rhinovirus infection leads to decreased TEER and increased permeability [19]. The BEAS-2B cell line, originally developed by immortalization of normal human bronchial epithelial cells using AD12-SV40 virus [25], has been less frequently used at ALI; however there is some literature using BEAS-2B in this system [17, 26]. Although these cells have been separately characterised by techniques such as immunofluorescence and TEER, no systematic comparison of primary cell and cell line culture models in this system has been reported.

The aim of the current study was to evaluate primary and non-primary bronchial epithelial cell culture systems as (ALI-) differentiated models for asthma research. We cultured primary HBECs from two donors, Calu-3, BEAS-2B, and BEAS-2B R1 (a subclone of BEAS-2B, cultured in the presence of foetal calf serum (FCS)) in their respective media at ALI. Development of TEER was measured over 28 days, RNA was collected at days 7, 14, and 21, and immunofluorescence was performed at day 28. We measured expression of a panel of differentiation (β-Tubulin IV, a ciliated cell marker, and MUC5AC, a goblet cell marker [27]) and tight junction/adhesion (E-Cadherin and ZO-1) markers by real-time quantitative PCR (qPCR) and confocal imaging to allow direct comparison of the phenotype of these different cell systems.

We show that although primary cells develop a differentiated phenotype, their TEER is highly variable, confirming the need to use multiple experiments and donors in primary cell systems. Calu-3 cells showed high TEER and similar expression of markers compared to primary cells, suggesting that these cells may be the most suitable model cell line for ALI experiments. Our work (1) indicates that all model systems, including primary cells, should be validated to ensure that the most suitable model is being used for a specific research question and (2) highlights the difficulties in utilising primary cells in epithelial cell research.

2. Materials and Methods

2.1. Cell Culture and ALI Differentiation. Human bronchial epithelial cells (NHBEC, Lonza, Wokingham, UK) were expanded in growth factor-supplemented medium (BEGM, Lonza) and differentiated at (ALI) at passage 3-4 in differentiation medium (BEDM) according to a previously published method [15, 28]. BEDM was composed of 50 : 50 Dulbecco's Modified Eagle's Medium (DMEM, Sigma) : BEBM (Lonza) with Lonza singlequots, excluding triiodo-L-thyronine and retinoic acid, but including GA-1000 (Gentamicin and Amphotericin-B). BEDM was supplemented with 50 nM retinoic acid at time of use. All experiments were performed using a single lot of BEBM and singlequots to avoid batch variation. Medium was used within one month of preparation, as recommended by Lonza.

Calu-3 lung adenocarcinoma cells [22] (obtained from ATCC) were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Sigma) supplemented with 10% FCS, 1% MEM non-essential amino acid solution (Sigma), and 1% Penicillin/Streptomycin (Sigma). BEAS-2B [25], a transformed bronchial epithelial cell line (gift from Dr R. Clothier, University of Nottingham), was cultured in BEGM and differentiated at ALI in BEDM.

Table 1: Antibodies used for immunofluorescent staining of cultured cells.

Target	Antibody	Dilution	Secondary
β-Tubulin IV	Sigma T7941	1 : 500	
MUC5AC	Abcam AB3649	1 : 250	Goat anti-Mouse FITC (Sigma F0257) 1 : 100
E-Cadherin	Millipore MAB3199Z	1 : 500	
Mouse IgG	Isotype Sigma M7894	1 : 463	
ZO-1	Invitrogen 40-2200	1 : 125	Goat anti-Rabbit Rhodamine TRITC (Stratech 111-025-003) 1 : 100
Rabbit IgG	Isotype Abcam AB27472	1 : 1	

Table 2: Primers and TaqMan probes used for qPCR assays.

Target	Primers	Probe
β-Tubulin IV	AGATCGTGCACCTGCAGG	CCAGTGCGGCAACCAGATCGG
	CATGGTATGTGCCTGTGG	
MUC5AC	TACTCCACAGACTGCACCAACTG	TGTGCTTGGAGGTGCCCACTTCTCAA
	CGTGTATTGCTTCCCGTCAA	
E-Cadherin	CCCACCACGTACAAGGGTC	CGAGGCTAACGTCGTAATCACCACACTGA
	CTGGGGTATTGGGGGCATC	
ZO-1	GCGGTCAGAGCCTTCTGATC	ACTCGCCGCAGCAGCCAAGCAAT
	CATGCTTTACAGGAGTTGAGACAG	

BEAS-2B R1 [29] (a subclone of BEAS-2B) (gift from Dr. R. Penn, University of Maryland, Philadelphia, PA) was cultured in DMEM supplemented with 10% FCS and 1% Penicillin/Streptomycin. All cells were cultured on 12 mm polyester Transwell inserts with a pore size of 0.4 μm (Corning NY, USA). Cells were plated at 100,000 cells per insert in appropriate medium. When confluent (~3 days), cells were raised to ALI. Medium was replaced and the apical face washed with phosphate-buffered saline (PBS) every 48 hours. RNA was extracted after 7, 14, and 21 days at ALI and cells were fixed for immunostaining after 28 days at ALI.

2.2. Transepithelial Electrical Resistance (TEER). The transepithelial electrical resistance (TEER) was measured in differentiating cells using an EVOM2 epithelial volt-ohm meter (World precision Instruments UK, Stevenage), over 21 to 28 days at ALI to confirm development of tight junctions. Briefly, medium was aspirated and replaced with 1 mL in the basolateral and 0.5 mL in the apical compartment. Cultures were equilibrated in the incubator for 30 minutes before measurement of TEER. Apical medium was then aspirated to restore ALI. TEER of insert and medium alone was subtracted from measured TEER and $\Omega \cdot cm^2$ calculated by multiplying by the insert area.

2.3. Immunofluorescence of Cultured Cells. ALI cultured cells were fixed *in situ* on inserts and transferred to glass slides for visualisation. Cells were fixed using 4% formaldehyde and blocked/permeabilised with PBS, 10% goat serum, 1% BSA, and 0.15% Triton-X. Cells were incubated with appropriate primary antibodies at 4°C overnight (Table 1), and FITC or rhodamine-TRITC labelled secondary for 1 hour at room temperature before mounting in HardSet DAPI (Vector Labs). Controls were incubated with secondary antibody alone or primary isotype control antibody followed by secondary antibody. Cells were visualized using the Zeiss spinning disk confocal microscope using Volocity software (version 5.5, PerkinElmer, Cambridge, UK).

2.4. Quantitative PCR (qPCR). Cultured cells were lysed and RNA was extracted using silica columns (RNeasy mini kit, Qiagen, Crawley, UK). cDNA was synthesized using Superscript II (Invitrogen, Paisley, UK) and random hexamer primers as per instructions. mRNA levels were quantified using a series of TaqMan assays (Table 2). Probes were labelled with FAM and TAMRA. qPCR was performed using TaqMan gene expression master mix (Applied Biosystems, Warrington, UK) and HPRT1 (4310890E, Applied Biosystems) endogenous control on a Stratagene MxPro3005 machine using 40 cycles of 95°C 15 sec, 60°C 60 sec. Data were normalised using the housekeeper (HPRT1) and the $2^{-\Delta Ct}$ method.

3. Results

3.1. Primary Epithelial Cells and Cell Lines Develop TEER When Cultured at ALI. Primary HBECs (2 donors) and cell lines were cultured at ALI and TEER measured every 2-3 days for 21–28 days (Figure 1, Table 3). All primary cell experiments were performed at passage 3-4 from different frozen vials. Experiments performed at passage three (two in Donor 1, one in Donor 2) developed TEER >350 $\Omega \cdot cm^2$, whilst experiments performed at passage four developed TEER <150 $\Omega \cdot cm^2$ (Figures 1(a) and 1(b)). Calu-3 (passage 35–37) developed maximum TEER >400 $\Omega \cdot cm^2$ in all experiments, reaching a peak between days 9 and 12. Thereafter, values

Table 3: A summary of outcomes for different cell types. For β-Tubulin IV and MUC5AC, "localised" expression refers to expression in a subset of cells. For E-Cadherin and ZO-1, expression was "localised" to the cell boundaries. *staining was similar to isotype control. HBEC D1 is Donor 1 and HBEC D2 is Donor 2.

		HBEC D1	HBEC D2	Calu-3	BEAS-2B	BEAS-2B R1
TEER		variable	variable	high	low	none
β-Tubulin IV	mRNA	low	low	high	mid	high
	protein	mid	mid	mid	high	mid
		localised	localised	diffuse	localised	localised
MUC5AC	mRNA	high	high	high	none	none
	protein	high	high	high	low	none
		localised	localised	localised	diffuse	
E-Cadherin	mRNA	high	high	mid	mid	none
	protein	high	high	high	mid	low
		localised	localised	localised	part localised	diffuse
ZO-1	mRNA	high	high	low	mid	mid
	protein	high	high	low	mid*	low*
		localised	localised	localised	part localised	diffuse

dropped slightly before reaching a more variable plateau (Figure 1(c)). BEAS-2B reached a maximum TEER of 100–150 $\Omega \cdot cm^2$ by around day 14 (Figure 1(d)), regardless of passage, whilst BEAS-2B R1 did not develop significant TEER (Figure 1(e)).

3.2. Primary Epithelial Cells and Cell Lines Show Morphological Differences. The different cells used in this study showed different phenotypes in culture. Phase contrast images give a limited indication of these differences; however gross morphological differences are present (Figure 2). Calu-3 cells took longest to become fully confluent, probably due to their tendency to form discrete colonies, unlike the other cells which form a more even monolayer. HBECs showed darker areas of denser (probably more stratified) cells and lighter, less dense areas (Figures 2(a) and 2(b)). The Calu-3 cell phenotype was more homogenous (Figure 2(c)), with increased mucus secretion apparent on washing. BEAS-2B cells consistently developed an apical layer of material which was not removed by washing (Figure 2(d)). BEAS-2B R1 cells had a very homogenous appearance, with no indication of mucus production or differentiation (Figure 2(e)).

3.3. Primary Epithelial Cells and Cell Lines Express Characteristic Differentiation Markers. At 28 days, cells were fixed and immunostained with antibodies specific for β-Tubulin IV and MUC5AC (Figure 3, Table 3). Although β-Tubulin IV is often expressed as a cytoskeletal protein, apical expression is a commonly used marker of ciliated epithelial cells [27]. MUC5AC is expressed by goblet cells as a component of mucus. Single image slices and z-stacks are shown to give an indication of the overall level of expression and location in the cell layer (basal versus apical). As ALI culture thickness varied between cell types, the brightest image is shown in each case. These were representative of 2-3 experiments per donor or cell type. HBEC images shown for both donors are from experiments reaching low TEER; however, β-Tubulin IV and MUC5AC expression did not seem to reflect TEER values (data not shown). HBECs presented apical β-Tubulin IV expression in a subset of cells with a greater proportion of cells from Donor 1 than Donor 2 showing expression. β-Tubulin IV expression was observed in Calu-3 layers but staining was only apparent below the apical pole of the cells. Strong staining for β-Tubulin IV was obtained at the apical side of BEAS-2B cells. BEAS-2B R1 showed apical staining in a subset of cells. While both HBEC donors and Calu-3 cells was stained positive for MUC5AC expression in a subset of cells towards the apical side of the cell layer, neither BEAS-2B subtypes showed significant MUC5AC staining.

3.4. Primary Epithelial Cells and Cell Lines Express Tight Junction Proteins. Sections were costained for expression of ZO-1 (a tight junction protein) and E-Cadherin (a cell adhesion molecule and epithelial cell marker). Matched, single confocal slices and z-stacks are shown. The brightest image from each stack was chosen to allow comparison of maximum expression in each cell culture system (Figure 4, Table 3). Images are representative of 2-3 experiments per donor or cell type. Both HBEC donors showed strong staining for ZO-1 that was localised to cell membranes/cell-cell junctions. This staining may be stronger in Donor 1 (where TEER reached >350 $\Omega \cdot cm^2$) than Donor 2 (where low TEER <150 $\Omega \cdot cm^2$ was reached); however the difference in staining was slight, compared to the variation in TEER. Overall, ZO-1 staining was performed in five HBEC experiments and no correlation between staining and final TEER was observed (data not shown). ZO-1 expression was weaker in Calu-3 cells, despite their consistently high (>300 $\Omega \cdot cm^2$) TEER, although similarly localised around cell boundaries. In both BEAS-2B subtypes, ZO-1 expression was generally diffuse; however, BEAS-2B cells showed membrane localised expression in the apical cell layer. Both BEAS-2B subtypes also showed high non-specific staining with the rabbit

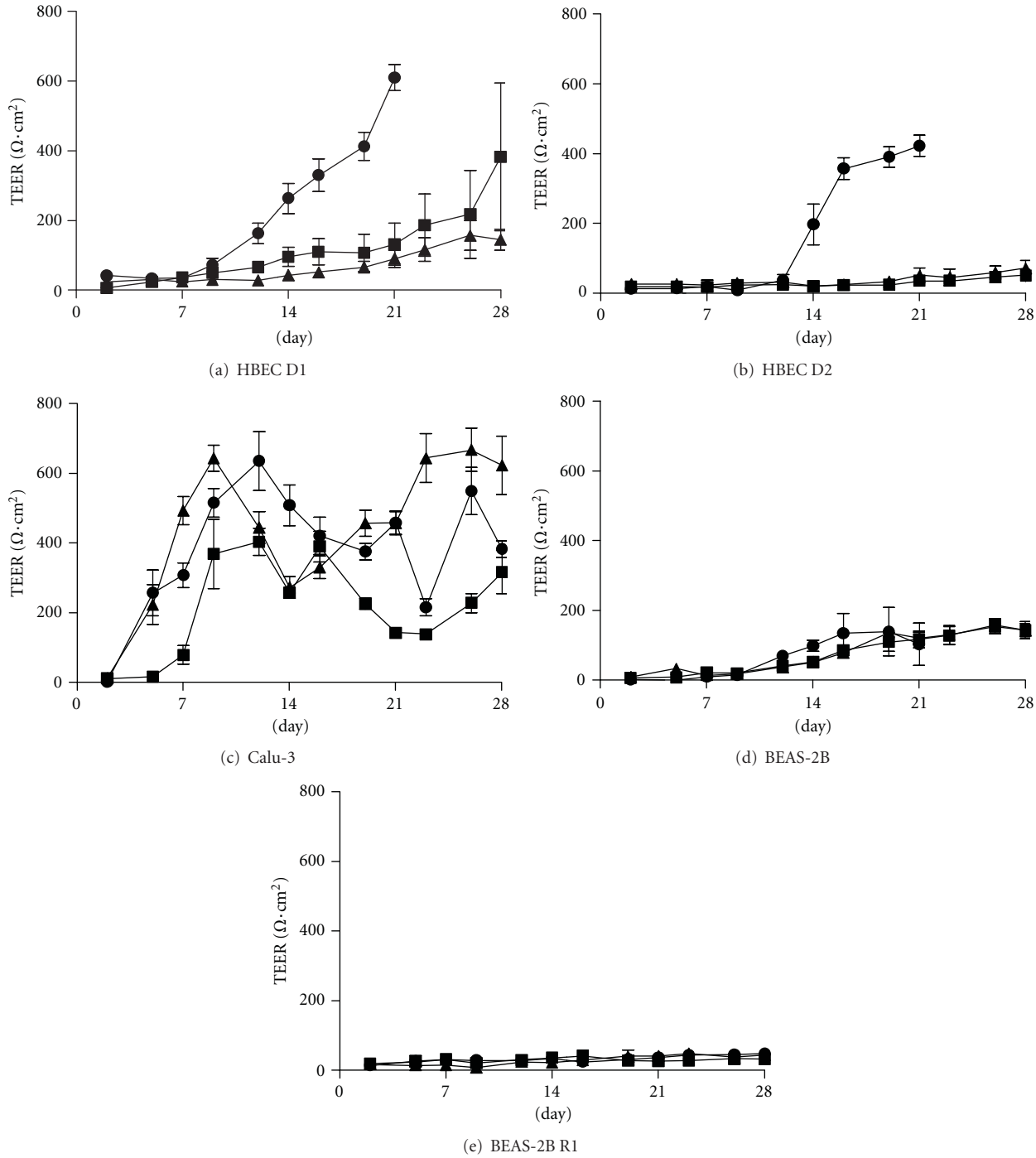
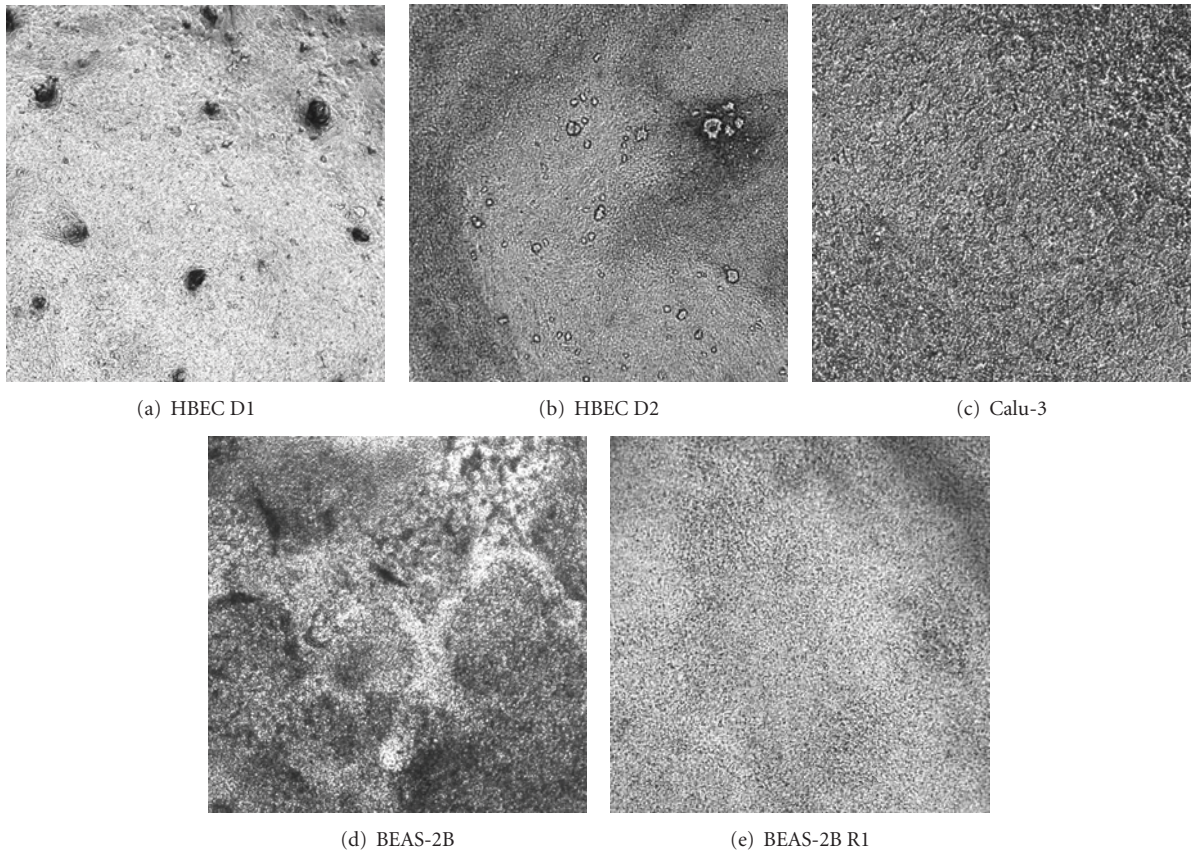


Figure 1: Development of transepithelial electrical resistance (TEER) in cells grown at (ALI). Different primary cells and cell lines were cultured at ALI over 21–28 days. TEER was measured every 2-3 days. Results from three separate experiments are shown for each cell line/donor, six replicates per experiment. HBEC D1 is Donor 1 and HBEC D2 is Donor 2. Error bars show standard deviation.

isotype control, suggesting that ZO-1 protein levels may be lower than they appeared. E-Cadherin expression was seen in all cells except BEAS-2B R1. In both HBEC donors and Calu-3, expression was tightly localised to the cell membrane/cell-cell junctions, whilst in BEAS-2B expression was more diffuse in the basal layer, but membrane was localised in the apical layer.

3.5. Expression of Differentiation and Tight Junction Markers Varies at the mRNA Level. Cells were harvested for RNA at days 7, 14, and 21 during ALI differentiation and qPCR performed for MUC5AC, β-Tubulin IV, E-Cadherin, and ZO-1 (Figure 5, Table 3). Representative data from one of two experiments are shown. HBEC results were taken from experiments in which TEER reached >350 $\Omega \cdot cm^2$ (Donor 1)



(a) HBEC D1 (b) HBEC D2 (c) Calu-3

(d) BEAS-2B (e) BEAS-2B R1

Figure 2: Phase contrast images of cells at ALI. Different primary cells and cell lines were cultured at ALI over 21–28 days. Phase contrast images were taken at 21 days. Representative images are from three independent experiments.

and low TEER (Donor 2), whilst in replicate experiments, TEER >350 $\Omega \cdot cm^2$ was reached in both donors. Expression levels of all genes were significantly different between cell types, although not between HBEC donors ($P < 0.001$ for all genes, 2-way ANOVA). These effects were conserved in a second independent experiment. Overall, no replicated trends in gene expression over time were observed.

MUC5AC mRNA was similar in HBEC and Calu-3 cells. Although expression of MUC5AC appears to increase at later time points in the experiment shown ($P < 0.001$, ANOVA), this effect was not conserved in a second independent experiment. MUC5AC mRNA was not detected in the two BEAS-2B subtypes (Figure 5(a)), consistent with immunofluorescence results. β-Tubulin IV expression was highest in BEAS-2B R1>Calu-3>BEAS-2B>HBEC (Figure 5(b)). This is in contrast to the immunofluorescence data where staining was lowest in BEAS-2B R1. Expression of E-Cadherin was highest in HBEC>Calu-3 and BEAS-2B>BEAS-2B R1 (not detected) (Figure 5(c)), whereas immunofluorescence was similar in HBEC and Calu-3. ZO-1 expression (Figure 5(d)) was highest in HBEC>BEAS-2B and BEAS-2B R1>Calu-3.

4. Discussion

We have evaluated two primary human donors of bronchial epithelial cells and Calu-3, BEAS-2B, and BEAS-2B R1 cell culture systems as ALI models of the airway epithelium for asthma research. For the first time, cell lines were directly compared to primary cells (Table 3). Using measurement of TEER [8], immunofluorescent staining, and qPCR, we have investigated formation of tight junctions (ZO-1 and E-Cadherin) as well as expression and localisation of suggested markers of ciliated (β-Tubulin IV) and goblet (MUC5AC) cells [27]. The main outcomes of our study are that (1) primary HBECs demonstrate a variable differentiated phenotype with the development of tight junctions and TEER showing experiment, passage, and donor variation, (2) Calu-3 cells exhibit many of the features of primary cells but have distinct differences including, for example, ZO-1 expression, and β-Tubulin IV localisation, although data generated were more reproducible, and (3) as anticipated, the BEAS-2B cell lines have limited differentiation capacity in ALI models. These data have implications for the use of both primary cells and cell lines for airway epithelial research in asthma.

The use of primary HBECs *in vitro* has provided insight into the potential mechanisms underlying asthma. This is exemplified by the recent findings of Xiao and colleagues [13], demonstrating that asthmatic epithelial cells at ALI show disrupted tight junctions and increased macromolecular permeability, reflecting the *ex vivo* phenotype. Another study by Hackett et al. observed an increased cytokine

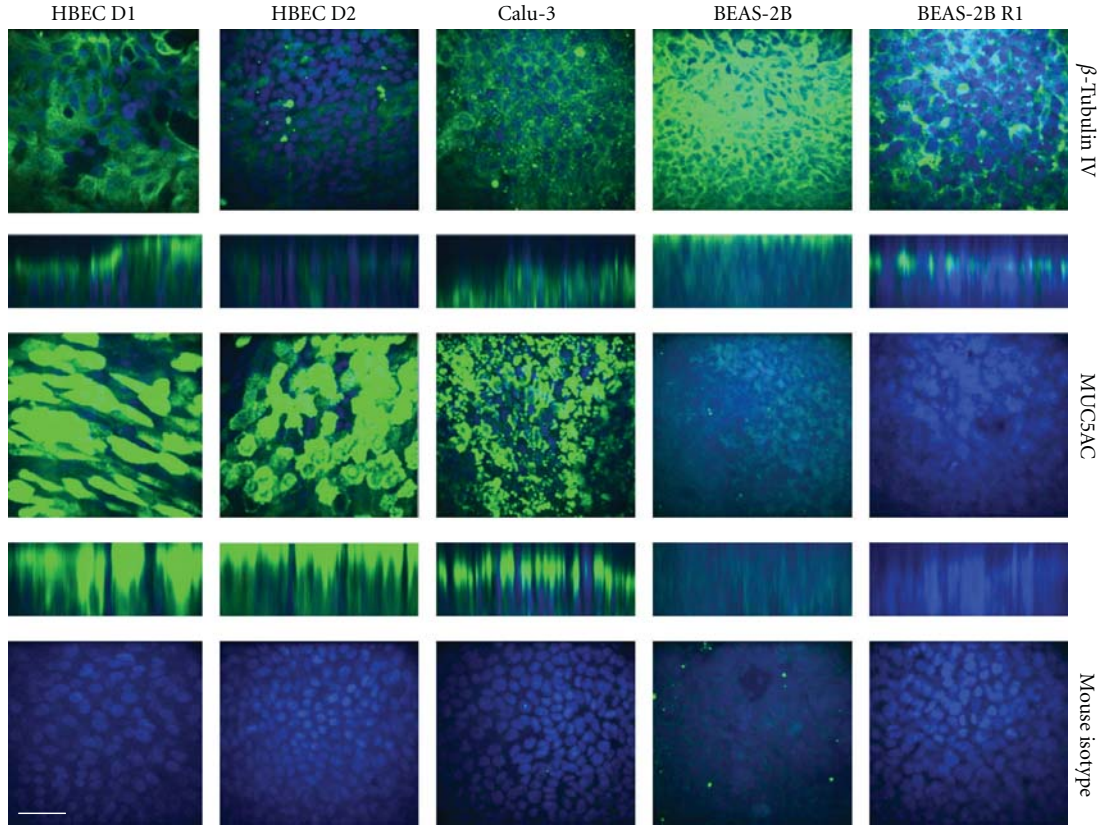


Figure 3: Immunofluorescent confocal imaging of differentiation markers. Localisation patterns of β-Tubulin IV, MUC5AC, and the Mouse IgG Isotype control at 28 days ALI were evaluated as described in the methods section. Single Z-slices are shown representing maximum intensity observed, with the corresponding Z-stack image below for β-Tubulin IV and MUC5AC. Scale bar represents 50 μm. Representative images are from three independent experiments. HBEC images were taken from experiments with low TEER (Figure 1).

response to particulate matter, viral exposure, or mechanical wounding [12], demonstrating that asthmatic cells may show an aberrant inflammatory response to common environmental stimuli. Primary and cell line systems also play a role in dissecting the signalling networks involved in asthma. Normal HBECs at ALI treated with T_H2 cytokines, for instance, show a potentially more secretory phenotype [15], whilst in HBEC or BEAS-2B cells, leukotriene D_4 signals via EGFR to release IL-8 [17]. It is beyond doubt that these epithelial ALI culture systems show utility in asthma research; therefore in the current study, we aimed to provide a direct comparison of a number of cell culture systems used in asthma research to help in selection of appropriate systems for specific research questions.

We measured expression of various proteins at the mRNA and protein levels as markers of differentiation. Although β-Tubulin IV is widely expressed in cultured cells, apical expression is often used to identify ciliated epithelial cells at ALI [27, 30], whilst MUC5AC is a mucus protein, expressed by goblet cells in the lung epithelium [31]. ZO-1 and E-Cadherin were included as markers of tight junction formation and barrier integrity. An alternative method of characterising ALI cultures is sectioning and performing histochemical analysis to confirm differentiation which gives a clearer indication of the multilayer structure (e.g. [12]). This study is limited by the use of immunofluorescence only; however we can obtain an overview of the phenotypes of different systems using this method.

In this study, we found that mRNA expression was not tightly linked to immunostaining, particularly for β-Tubulin IV, where HBECs showed very low mRNA levels but high protein expression. This suggests that mRNA expression may not be a good marker of functional status for these genes. Differences between mRNA and protein levels may reflect experimental or biological issues [32, 33]. In our study, samples were taken for qPCR at days 7–21 and for immunofluorescence at day 28, a limitation which may partially explain these differences. Some variation in immunofluorescence between samples may reflect different protein localisation; that is, diffuse faint staining may reflect similar amounts of protein to bright, localised staining. At the biological level, differences between mRNA and protein may reflect variation in posttranscriptional mechanisms between the cell types, such as mRNA stability or protein synthesis and turn-over.

When culturing primary cells at ALI, the choice of medium is very important, with different media delivering different degrees of stratification and cell phenotypes [5, 34]. We use "Gray's medium" (BEDM), which is reported to allow development of a pseudostratified, polarised phenotype,

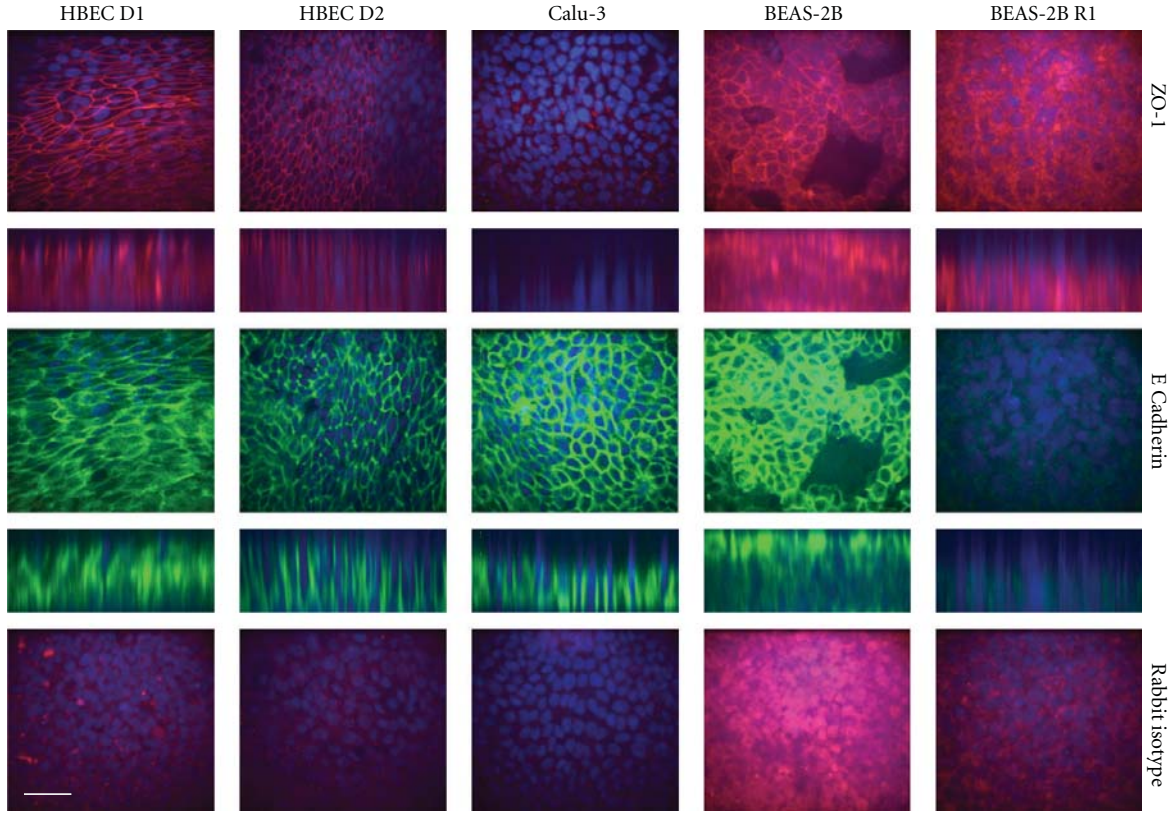


Figure 4: Immunofluorescent confocal imaging of tight junction proteins. Localisation patterns of ZO-1, E-Cadherin, and the Rabbit IgG Isotype control at 28 days ALI were evaluated as described in the methods section. Images shown are single Z-stack slices representing maximum intensity observed with the corresponding Z-stack image below and are of matched fields using dual staining. The corresponding Mouse Isotype control for E-Cadherin can be seen in Figure 3. Scale bar represents 50 μm. Representative images are from three independent experiments. Images for HBEC Donor 1 were taken from an experiment reaching high TEER (>350 $\Omega \cdot cm^2$), whilst Donor 2 images were taken from an experiment reaching low TEER (Figure 1).

including ciliated and goblet cells. This was confirmed in our hands, with localised expression of E-Cadherin, MUC5AC, and β-Tubulin IV observed in both primary cell donors. This model is reported to develop TEER [6, 7]. We found that development of TEER was variable. TEER >350 $\Omega \cdot cm^2$ was obtained in the three experiments performed at passage three, whilst TEER <150 $\Omega \cdot cm^2$ was obtained at passage four, despite consistent expression of ZO-1 mRNA and protein, localised to the cell membrane/cell-cell junctions. These observations reinforce the assumption that localised ZO-1 staining is not a surrogate marker for TEER and *vice versa*, as well as the importance of passage when using primary cells. The variation seen in this study between different experiments in a single donor is indicative of the potential issues when comparing normal *versus* asthma cells. Routinely, a single experiment is performed per donor [12, 13]. It is important that these experiments are performed with cells cultured for the same period of time and in the same batch of medium to minimise experimental variation.

The Calu-3 cell-line was established from a pleural effusion of a lung adenocarcinoma, derived from submucosal gland serous cells [22–24]. Calu-3 cells are routinely cultured in FCS-supplemented media [23] and spontaneously differentiate at ALI to give significant TEER [23, 35]. These cells are reported to express ZO-1 (a tight junction protein) and E-Cadherin (an epithelial marker and cell adhesion protein). We showed development of TEER >400 $\Omega \cdot cm^2$ and some expression of ZO-1 and E-Cadherin. Interestingly, although TEER was higher and more robust than in the primary HBECs, ZO-1 expression at both the mRNA and protein levels was lower, demonstrating that other tight junction proteins have a role to play in maintaining TEER. The cells expressed apical MUC5AC, as anticipated from their known secretory phenotype. However, staining for β-Tubulin IV expression was diffuse and not clearly located at the apical side, suggesting that villi or cilia had not formed in the Calu-3 model, in accordance with previous studies [23] that have shown that ciliated cells are sparse in the Calu-3 cell line.

The BEAS-2B cell line was originally developed by immortalization of normal human bronchial epithelial cells using AD12-SV40 virus [25]. This parental population of cells (as well as subclone S6, not used here) retains the ability to undergo squamous differentiation in response to TGFβ1 or serum [29]. The BEAS-2B R1 line was derived from the parental population by subculture in the presence of 5%

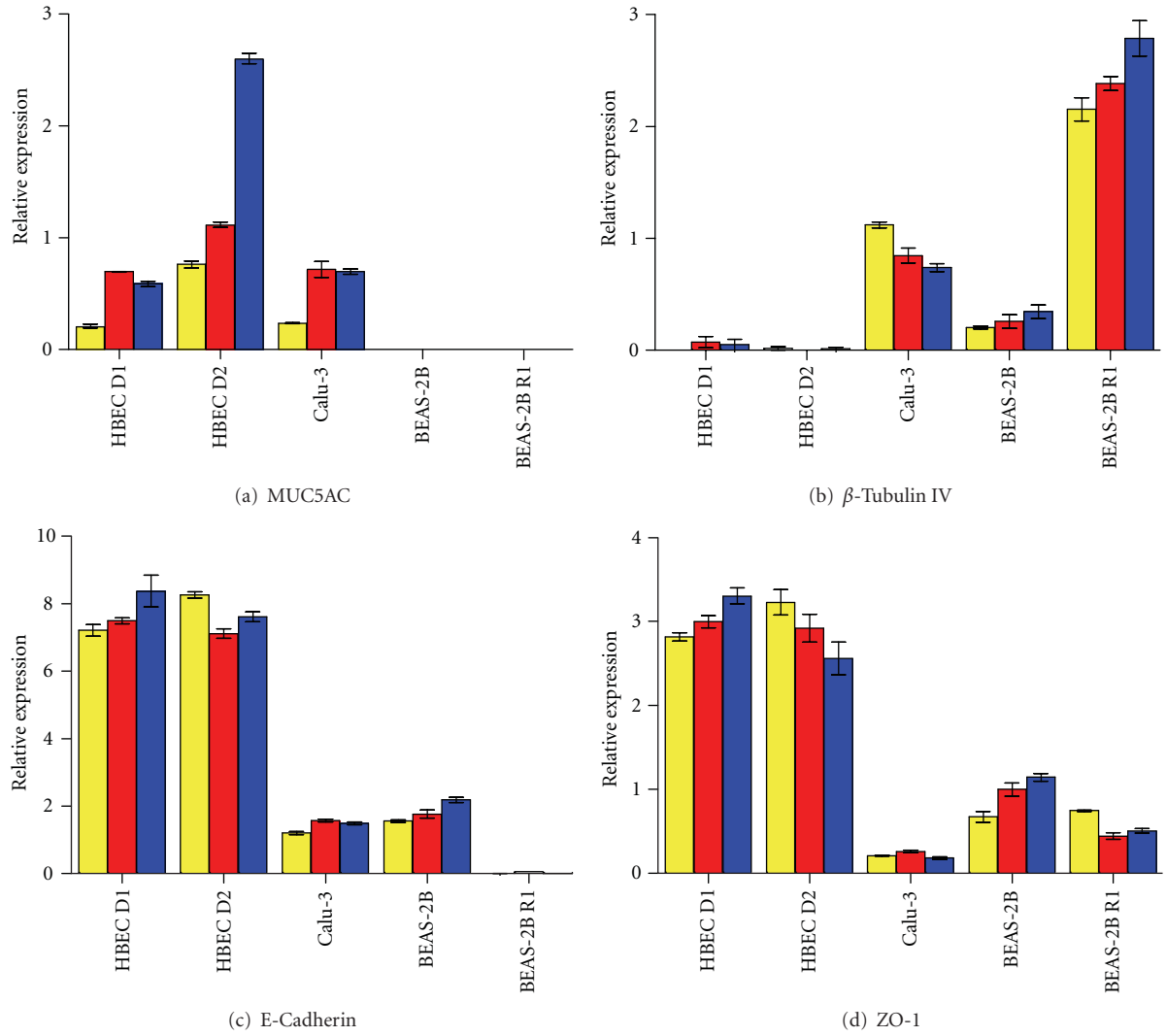


Figure 5: mRNA expression of differentiation and tight junction markers. Primary cells and cell lines were cultured at ALI over 21–28 days. RNA was extracted at days 7, 14, and 21 during ALI differentiation for each cell line or donor. Expression of MUC5AC (a), β-Tubulin IV (b), E-Cadherin (c), and ZO-1 (d) was measured. Data are normalised to the housekeeping gene HPRT1. Data are representative of two independent experiments. Error bars show standard deviation. Yellow, red, and blue bars represent expression at days 7, 14, and 21 post-ALI, respectively.

FCS. Unlike the parental cell line, these cells are induced to proliferate by serum or TGFβ1 and have a more fusiform appearance [29]. BEAS-2B cells (S6 subclone, similar to the parental population) have previously been shown to attain TEER >100 Ω·cm^2 at higher passage, in KGM (keratinocyte growth medium, Clonetics) when supplemented with calcium [26], or when grown in BEGM [36] or Laboratory of Human Carcinogenesis (LHC) serum-free medium [37]. We used BEDM to drive BEAS-2B towards a differentiated phenotype. These cells attained the reported TEER of >100 Ω·cm^2. At ALI, these cells developed an apical layer which stained strongly for β-Tubulin IV and showed localised ZO-1 and E-Cadherin staining. However, staining was fainter and more diffuse in the basal layer. It may be the presence of this apical layer that increases the TEER, rather than tight junction formation throughout the culture model. These cells did not express MUC5AC.

There is no literature regarding the use of the BEAS-2B R1 cell line at ALI; therefore these cells were included essentially as a negative control, cultured in DMEM with 10% FCS. As anticipated, these cells did not develop TEER and expressed minimal levels of E-Cadherin at the RNA and protein level. The cells show apical β-Tubulin IV expression, but no MUC5AC or localised ZO-1 expression. The reduced E-Cadherin expression of this cell line suggests that they may have developed a more mesenchymal phenotype by culturing in the presence of FCS.

5. Conclusions

Normal and asthmatic primary bronchial epithelial cells and cell lines are widely used in asthma research. ALI models are used to attempt to more closely replicate the *in vivo* situation.

We have evaluated primary bronchial epithelial cells from two donors and three cell lines in an ALI model with respect to various markers of differentiation. Although primary cells are regarded as the most physiologically relevant, they exhibit a high degree of variability between donors, experiments, and passage, particularly with respect to development of TEER. Primary cells are costly and therefore unsuitable for large scale experiments such as drug screening; they also have a finite lifespan and may be difficult to manipulate. Cell lines may, therefore, present an attractive alternative model. We found that Calu-3 cells develop a high TEER and have a pattern of expression of epithelial markers similar to primary cells. Although frequently used in monolayer culture, the two BEAS-2B cell lines did not perform well in the ALI model, showing poor TEER and lacking expression of epithelial differentiation markers.

This work underlines the importance of using a well-characterised model, suitably validated for the outcomes of interest in any experiment. Importantly, this study highlights some of the challenges ahead characterising primary human airway epithelial cells from asthma and control donors accounting for the inter- and intradonor variability identified in the current study.

Acknowledgments

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Review Article

Apoptosis and the Airway Epithelium

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The airway epithelium functions as a barrier and front line of host defense in the lung. Apoptosis or programmed cell death can be elicited in the epithelium as a response to viral infection, exposure to allergen or to environmental toxins, or to drugs. While apoptosis can be induced via activation of death receptors on the cell surface or by disruption of mitochondrial polarity, epithelial cells compared to inflammatory cells are more resistant to apoptotic stimuli. This paper focuses on the response of airway epithelium to apoptosis in the normal state, apoptosis as a potential regulator of the number and types of epithelial cells in the airway, and the contribution of epithelial cell apoptosis in important airways diseases.

1. Introduction

The airway epithelium is the first barrier and first line of host defense in the airway. Formerly considered a more inert barrier that "kept the outside out and the inside in," it is now clear that epithelial cells participate in host defense and inflammation. The networks in which the epithelium participates indeed can orchestrate either or both, depending on whether these networks are activated normally or not.

A more classical view of epithelial responses to injury and inflammation emphasized the ability of the epithelium to respond to insults by secretion of water and mucous into the airways and mediator secretion (e.g., cytokines and chemokines) into the local environment and into the circulation. In this view, the epithelial layer responded to physical injury by a process that included, in order, phagocytic clearance of damaged cells and material, proliferation of new epithelial cells from surviving nearby stem cells, differentiation (phenotype shifting may be preferred) to new, required cell subtypes such as ciliated and mucous (goblet) cells, and restoration of barrier function [1]. Over the past two decades, it has become clear that proper protection and repair of the airway mucosa against sustained damage may also depend on the processes that control programmed cell death, that is, apoptosis.

Apoptosis is a tightly regulated process of nonnecrotic cell death that is critical for normal tissue and organ homeostasis. Cells undergo apoptosis through the activation of carefully regulated pathways that lead to their orderly shutdown and removal. In this paper, I examine the occurrence and function of apoptosis both in the normal airway epithelium and in the epithelium in several airways diseases. In the context of these diseases, epithelial cell apoptosis may be either a compensatory response, a pathogenetic consequence, or both. I limit this paper to discussion of central airways (tracheal and bronchial) epithelial cells with appropriate mention of small airway and alveolar epithelial cells where warranted and to the process of apoptosis, leaving aside other mechanisms of cell death such as necrosis and autophagy.

2. Studying the Airway Epithelium in Apoptosis

In examining apoptosis in the airway epithelium, it should be noted that several methods examine epithelium both *in situ* and in culture. Each of course has its limitations and strengths.

Collection of full-circumference airways is useful to examine epithelial morphology, damage, proliferation, and apoptosis in a setting that preserves the architecture of

the full-thickness airway. Various morphologic and histologic strategies, including antibody labeling or electron microscopy, can be used. This method is limited to the study of specimens collected either by lung resection or at autopsy. Endobronchial biopsies can be obtained more easily (compared to open lung biopsy or autopsy) and allow for both one-time and, in some special cases, repeated sampling of airways, but crush artifact may limit the ability to interpret morphological changes in the mucosal layer [2]. Endobronchial brushings can collect epithelial cells for subsequent culture or harvest of RNA but provide no information about morphology.

The culture of airway epithelial cells likewise can be useful to elucidate mechanisms but has limitations. Primary cells typically have been grown in submersion culture, a method used for over four decades in many laboratories; these have a monomorphic appearance that resemble basal airway epithelial cells. Submersion culture can generate experiments quickly to test hypotheses and mechanisms but do not provide information about the morphology of epithelial subtypes and thus raises a concern about how applicable the results from these experiments are to the in situ state. Epithelial cells can be grown in an "air-liq-uid interface" (ALI), a more recent culture method that permits differentiation (a better term may be "phenotype shifting") over a period of 2 to 5 weeks of culture into basal, columnar, and goblet cells that resembles a native epithelium [3, 4]. While more technically demanding, such cultures provide additional information about morphology and potential interactions between the epithelial subtypes. As with submersion culture, data from cells grown in ALI culture may not be fully applicable to the *in situ* airway. Finally, a number of airway epithelial cell lines have been generated over the past three decades. Several of these, such as the 16HBE14o- and 1HAEo- cell lines that are SV40 transformed from normal cells [5, 6] and the A549 lung adenocarcinoma cell line [7], have been popular in studies of epithelial cell apoptosis due to the ease of culture and the reproducibility of the cells over a number of passages. However, whether SV40 transformed or derived from cancer cells, it should be acknowledged that proliferation and apoptosis pathways and regulators may well be different in these cells versus primary cells and that cells in culture may not behave as epithelial cells *in situ* in their unique microenvironment.

One potential issue with the culture of epithelial cells is that (done properly) there are no other cell types in the culture system. While the response of pure epithelial cells to a stimulus is useful in reductionist-style experiments, examining interactions between different cell types may be just as important. To meet this concern, dual-culture systems grow epithelial cells on a filter either in submersion or in air-liquid interface suspended above a second cell type (e.g., fibroblasts) grown either on the underside of the filter or in the bottom of the culture container. Other dual-culture systems expose epithelial cells to bacteria (e.g., coculture or addition of *Pseudomonas aeruginosa*), with subsequent examination of apoptotic markers. Such biculture systems permit a limited examination of influences of these cells on epithelial cell apoptosis, or vice-versa.

Understanding the role of apoptosis in airway epithelium and interpreting the many studies done also require a clear awareness of the usefulness and limitations of each method of analysis. A recounting of apoptosis methods is well beyond this paper but two recent reviews outlining guidelines for the use and interpretation of assays to analyze cell death (apoptosis, autophagy, and necrosis) are useful reminders of the significant methodological and analytical issues [8, 9].

3. Apoptosis Mechanisms

Apoptosis is the orchestration of cell death by highly conserved genes in eukaryotes [10–13] that permits the removal of damaged or unneeded cells from tissue without releasing cellular contents that would otherwise damage surrounding cells or elicit an inflammatory response. It is tightly and carefully regulated in normal circumstances but may be inappropriately activated or suppressed in diverse disease states [12]. In contrast to cell necrosis, the principal features of apoptosis include shrinkage of the cell, condensation of the nucleus, DNA fragmentation, fragmentation of the cell cytoskeleton, and eventual formation of an apoptotic body that is either shed or phagocytized [14]. Several reviews examine general mechanisms of apoptosis and are recommended [9, 12, 15–17].

Multiple signals that initiate apoptosis including death receptors, cytokines, heat and oxidative stress, and ionizing radiation link to a signaling cascade of serine proteases known as caspases. These proteases are constitutively expressed as inactive zymogens that are cleaved when activated. Upstream caspases (e.g., caspase-8) work to collect receptor-mediated signals and initiate the death cascade, whereas downstream caspases (e.g., caspase-3) act upon targets in the both cell nucleus and cytosol (Figure 1).

Two distinct pathways exist for caspase activation. The first, type I, termed the "extrinsic" pathway because it is activated by extracellular signals, involves ligation of cell surface death receptors [11, 13, 18, 19], members of the tumor necrosis factor (TNF) superfamily, such as CD95 (also Fas or APO-1) [14, 20], TNFR1, DR3, DR4, DR5, DR6, EDAR, and p75NTR [21]. Each contains an exclusive, 80 amino-acid long domain, the "death domain," essential to induce apoptosis [13, 21, 22]. When ligated, the receptors form homotrimers that recruit molecules into a "death domain." Activation of these domains (e.g., interaction of Fas with Fas-associated death domain or FADD, or the interaction of TNFR1 with the TNR-receptor-associated death domain or TRADD) leads to formation of a death-inducing signaling complex (DISC) [23, 24] and subsequently caspase activation [25, 26].

The cognate death receptor ligands generally are comprised of a C-terminal extracellular portion which interacts with the death receptor, a transmembrane region, and an N-terminal domain. The best-described ligands are CD95L (FasL), that bind CD95, TNF-α, and the TNF-related apoptosis-inducing ligand (TRAIL).

CD95L is perhaps the best-characterized death receptor ligand. It was originally considered to be expressed mostly

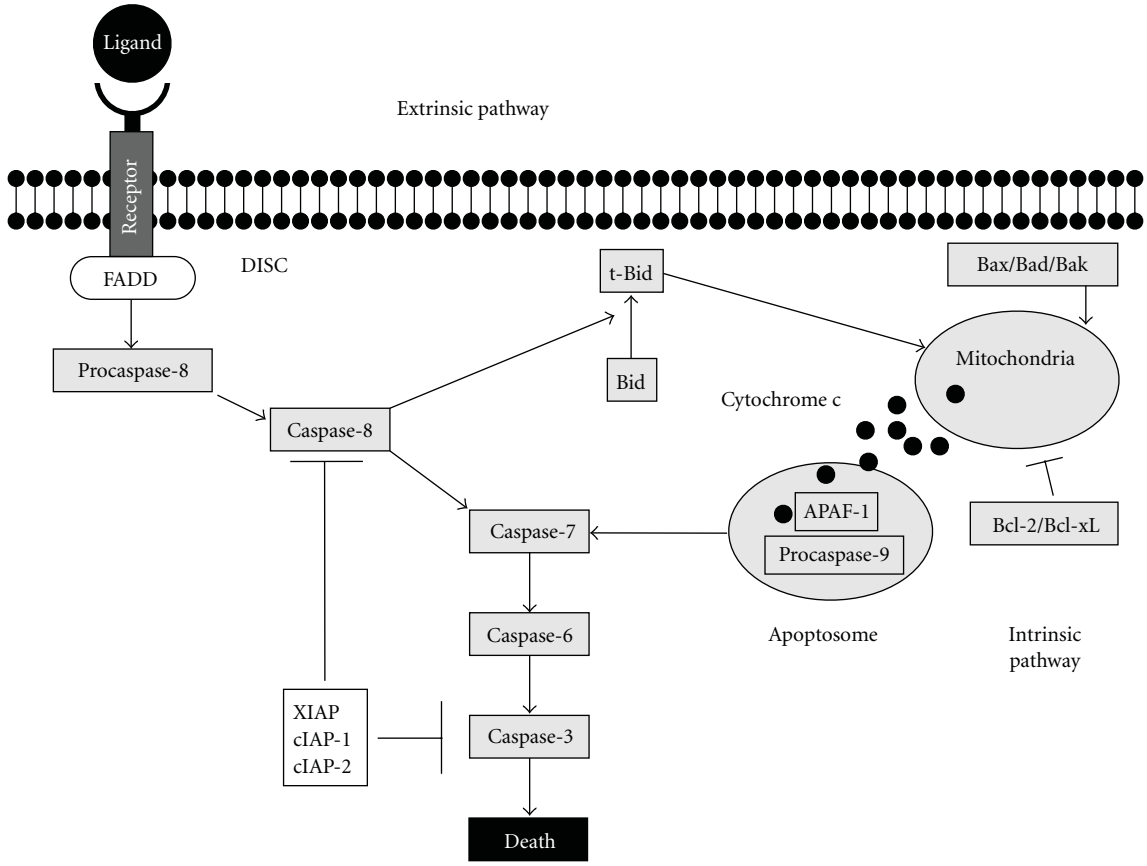


Figure 1: Extrinsic (receptor-mediated) and intrinsic (stress or oxidant-mediated) pathways that initiate apoptosis. Receptors such as CD95 and DR4 initiate apoptosis by interacting with death domain proteins such as FADD or TRADD, leading to the cleavage and activation of procaspase-8 with subsequent activation of downstream caspases and the initiation of the nuclear and cytoplasmic events that comprise apoptosis. In the intrinsic pathway, stress or oxidant-mediated injury leads to activation of proapoptotic BH3 proteins such as BAX or BAK; these then induce disruption of mitochondrial polarity leading to the release of cytochrome c, which binds APAF-1 in the "apoptosome," leading to cleavage and activation of caspase-9. This then leads to downstream caspase activation. Cross-talk from the extrinsic pathway via the truncation of Bid (to t-Bid) can also elicit disruptions of mitochondrial polarity so that both pathways may be activated. Mitochondrial integrity is regulated by a series of related antiapoptotic (Bcl-2, Bcl-xL) and proapoptotic (Bad, Bax, Bak) proteins. The downstream caspase cascade can be inhibited by inhibitors of apoptosis such as XIAP, cIAP-1, and cIAP-2.

by hematopoietic cells such as lymphocytes and dendritic cells, but also is demonstrated in immune-privileged sites and in states of chronic inflammation, and not only mediates cell death but also serves as an effector molecule to establish immune privilege and enhance cell survival [21, 27]. Originally described as a transmembrane molecule, CD95L now is recognized to have a soluble variant cleaved from the cell surface by metalloproteases [28, 29]; alternately, it is packaged with other lysosomal proteins in lysosomes and transported to the plasma membrane where it is then released into the external environment [27]. Soluble CD95L binds its receptor with the same efficiency as the transmembrane form.

TNF-α has complex effects on airway epithelial cells and may both elicit and oppose apoptosis depending on context. It is a "proinflammatory" cytokine that induces several effects in airway epithelium, such as (of many examples) the expression of the ICAM-1 adhesion molecule [30–32] and IL-6 and IL-8 [33–35]. TNF-α binds two distinct receptors: TNFR1 and TNFR2 [36]. The former is responsible for the cytotoxic, including apoptotic, effects of TNF-α, whereas both receptors can interact with a series of adaptor proteins, the TNF-associated factors (TRAF). TRAF2 but not TRAF1 can initiate activation of the NF-κB signaling cascade [37] to elicit IL-8 secretion [33, 34]. Disrupting NF-κB signaling can enhance TNF-α-mediated apoptosis; likewise, activating NF-κB signaling can suppress TNF-α-mediated apoptosis [38]. Thus, TNF-α-mediated cell death and inflammation exist in a balance that can be perturbed by several pathways.

TRAIL also is a member of the TNF superfamily [39]. TRAIL is a type II membrane protein that induces apoptosis in a variety of target cells. In some cell types, TRAIL binding to the death receptors DR4, DR5, and/or DcR2 may also activate the transcription factor NFκB, leading to transcription of genes that actually antagonize death signaling pathways and promote inflammation [40, 41].

A second pathway that elicits apoptosis, type II or the "intrinsic pathway," is activated not by receptors but

by stressors such as oxidative stress, DNA damage, and starvation [14, 42] and has as its defining characteristic a requirement for increased mitochondrial permeability that then releases cytochrome c. Cytochrome c combines with APAF-1 to cleave and activate procaspase-9 in a complex termed the "apoptosome" [19, 43, 44]. The mitochondria thus may serve as a "central apoptotic executioner" to integrate multiple, diverse internal signals that indicate cellular damage. Activation of either pathway leads to cleavage and activation of common pathway caspases such as caspase-3 (Figure 1) and commits the cell to an apoptotic death [14].

Both pathways are regulated by a family of proteins known as the inhibitors of apoptosis (IAPs). Most of these suppress caspases either by binding the active catalytic site of effector caspases, by preventing dimerization of caspase-9, by sequestering mitochondrial proteins, or by stimulating degradation and ubiquitination of caspases [45]. The Bcl-2 family of prot1ns also heavily regulates the type II mitochondrial pathway. These proteins have a conserved Bcl2 homology domain that allows the family member to join to other members. The prosurvival members of the family (Bcl2, Bcl-XL, BclW, MCL1, A1, and BOO/DIVA) have multiple Bcl2 homology domains [14, 46]. In contrast, the proapoptotic protiens have a different number of Bcl2 homology domains: BAX and BAK, for example, each has three such domains, and both function to increase mitochondrial membrane permeability and thereby release cytochrome c [14, 47]. Other proapoptotic Bcl proteins (BIM, BID, PUMA) have an alternate Bcl2 homology 3 (BH3) domain that binds to and inhibits prosurvival Bcl2 family members, thus releasing BAX and BAK [48, 49]. A balance between prosurvival and proapoptotic Bcl2 proteins determines life or death, and each family member may be activated or suppressed differently depending on the cell stress or death activator.

4. Apoptosis Receptors and Receptor Ligands in Epithelial Cells

One death receptor clearly identified on airway epithelial cells is CD95. Our laboratory group first demonstrated this death receptor and the expression of its corresponding ligand CD95L (FasL), in primary central airway epithelial cells and cell lines in submersion culture and in airways collected from lung resection surgery [20] (Figure 2). Ligation of CD95 using the Ch11 antibody that cross-links the receptor activates caspase pathways and elicits cell death [20, 50, 51]. Both CD95 and CD95L subsequently have been demonstrated in airway epithelium in patients with cystic fibrosis [52]; CD95L expression is markedly increased in these airways, which was also demonstrated in an epithelial cell line, HTEC, with a CF genotype. These observations, including that the CD95 receptor is functional, have since been replicated; one such study suggests that epithelial cell apoptosis induced by CD95 ligation may be less important than that induced by TNF-α [53]. CD95 has been demonstrated in small airway epithelial cells (SAEC) in culture, and when compared to epithelial cells collected from larger, proximal airways, SAEC is more sensitive to CD95 ligation with a recombinant, soluble FasL [54]. CD95 expression also has been demonstrated in type II alveolar epithelial cells in humans and other mammals [55–58] and may be involved in fetal lung development [59].

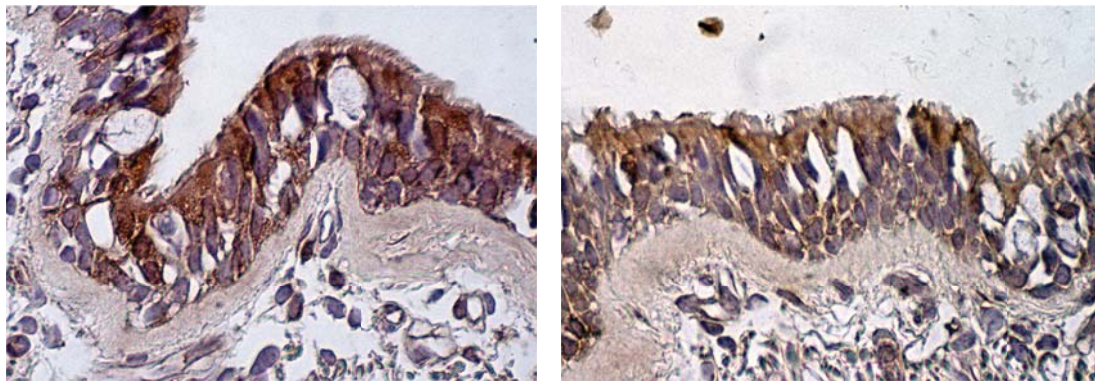
CD95L expression in human airway epithelium may serve, as it is postulated to serve in other immune privileged sites such as retina, brain, and testis, as an "immune barrier" [20, 60]. Airway epithelial FasL levels are increased in patients with severe asthma after steroid treatment, although it is possible that this increase in FasL levels reflects a more severe stage of disease [61]. One recent paper demonstrates that transmembrane FasL on epithelial cells is cleaved by matrix metalloproteinase (MMP)-7; this in turn is upregulated by the Th2-associated cytokine IL-13 [62]. As this MMP is increased in asthmatic airway epithelium, this study suggests a mechanism whereby increased CD95L in asthmatic airways might serve either to reestablish the immune barrier (by activating apoptosis in inflammatory cells) or to perpetuate mucosal damage (by activating apoptosis in epithelial cells).

These studies, taken together, make clear that both CD95 and its ligand are present and functional in airway epithelium both *in vivo* and in culture models and may be increased in expression in asthmatic epithelium.

As noted previously, TNF-α binds two distinct receptors: TNFR1 (also p60 TNFR) and TNFR2 (p80 TNFR). The former has an 80 amino-acid cystein-rich domain in its extracellular domain that can contain either a death domain, a TRAF binding domain, or a decoy domain [63]. The death domain interacts with the TNFR-associated death domain protein (TRADD), which in turn interacts with the Fas-associated death domain protein (FADD) to activate caspase-8 [37, 64]. In this way, TNFR1 shares the same downstream signaling pathway and machinery as CD95.

Remarkably few studies have been done to examine TNFR-pathway-driven apoptosis in airway epithelial cells and cell lines. Mitola et al. examined the effect of sputum sol phase collected from a cohort of patients with cystic fibrosis on apoptosis of primary human bronchial epithelial cells [65]. The TNF pathway was activated, but a direct link to TNF-α in the sputum sol phase was not demonstrated. Another recent study demonstrated that TNF-α stimulated caspase-3 activation and IL-8 expression in primary airway epithelial cells via phosphorylation of p38 mitogen-activated protein kinase (MAPK), an effected potentiated by concurrent exposure to nontypeable *Haemophilus influenzae* [66]. Activation of NF-κB pathways and involvement of TRADD were not examined in this study. Other stress-activated protein kinases such as c-Jun N-terminal kinase (JNK) are well recognized to suppress TNF-α-stimulated apoptosis (reviewed in [67]), but this has not been studied in airway epithelium.

In addition to receptor-driven activation that directly initiates apoptosis, TNFR activation may modulate apoptosis by indirect mechanisms, in addition to any survival signals and inflammatory signals delivered by its activation of the NF-κB signaling pathway. For example, treatment with TNF-α in primary proximal airway epithelial cells enhances subsequent apoptosis elicited by exposure to



(a) (b)

Figure 2: Expression of the CD95 receptor (a) and its ligand, CD95L (b), in a normal human airway using an immunoperoxidase method. Both basal and columnar cells stain for receptor and ligand (brown, with hematoxylin counter label). The appropriate controls (not shown here) for substitution of primary antibody do not label. Original magnification, 200x. From [20].

CD95L [54], demonstrating a clear potential interaction in these pathways. TNF-α treatment in the H441 and A549 lung adenocarcinoma lines elicits gene expression of both *TRAF1* and *cIAP2* and that both were increased in the lungs of infants with fatal bronchopulmonary dysplasia [68]. These signaling interactions are complex but suggest potential therapeutic strategies in manipulating death versus survival pathways in TNFR-mediated signaling.

The expression of TRAIL, a ligand for several death receptors, is increased, as demonstrated by immunohistochemical labeling, in the epithelium of endobronchial biopsies collected from asthmatic airways [69]. TRAIL expression is also noted in Th2 cells [70]. However, in contrast to the clear presence of CD95, no published studies have demonstrated the presence of potential partner death receptors, including DcR1, DcR2, DR4, or DR5, in airway epithelium in normal human airways, though one paper demonstrates both DR4 and DR5 in guinea pig airways [71], and one paper demonstrated the R1 and R2 receptors for TRAIL in nonbronchoscopically obtained epithelial cells from children suffering from RSV infection accompanied by respiratory failure and mechanical ventilation [72]. TRAIL may bind one of the TNFRs that are induced during diseases processes, which then may activate cell death pathways via relatively novel signals that include phosphorylation of p38-MAPK [66]. The relative expression of and signaling pathways connected to these receptors and the circumstances of their expression in health and in airways diseases in human airway mucosa require further exploration.

5. Apoptosis in Normal Airway Epithelium

Apoptosis has an important, beneficial regulatory role in the normal airway epithelium. As noted previously, it provides a mechanism to remove damaged cells without provoking an inflammatory response. An additional beneficial role is, along with cell proliferation, regulating the number of epithelial cells. The cell cycle rate in resting mammalian large airway epithelium *in situ*, expressed as the proportion of dividing cells counted in thymidine incorporation assays, is <1% [73, 74]. Corresponding rates of apoptosis, as judged by labeling for an apoptosis marker (e.g., TUNEL), are also low in normal mouse [75] and human [76, 77] airway mucosa. Exogenous and endogenous signals that stimulate proliferation may also stimulate apoptotic pathways so as to counter survival signals and thus maintain epithelial cell homeostasis [78].

One potential problem in measuring rates of apoptosis in airway epithelium, either normal or in disease states, is that dying epithelial cells may slough into the airway lumen prior to the demonstration of classic apoptosis markers by morphological methods. Detachment followed by cell death, termed *anoikis* (for a review see [79]), may lead to underestimation of rates of apoptosis in airway epithelium, though other markers of damage, such as focal gaps, denudation, or expression of markers such as the epithelial growth factor receptor or c-erbB-2 [80, 81], are still present. Dead (apoptotic or necrotic) epithelial cells in sputum degrade rapidly and may not be recognized or counted. One recent study collected sputum from marathon runners and then examined the number of apoptotic epithelial cells by TUNEL labeling followed by histologic examination. Their study demonstrated an ability to detect a significant number of positive cells [82]. This study suggests technical feasibility to examine the sputum. Another study examined the expression of *DAP kinase*, noted to have a role in TNF-α, Fas, and interferon-gamma- (IFN-γ-) induced apoptosis [83, 84] and in oncogenic transformation of cells [85], and in epithelial cells present in the sputum of a cohort of cancer patients, and correlated this to changes in expression in airway epithelium collected by bronchial brushing [86]. This study suggests that surrogate markers might be used to examine the presence of apoptotic epithelial cells and to examine activation or expression of pathways that might initiate cell death.

Another difficulty of detecting apoptotic cells in either normal or diseased tissue underscores the purpose of apoptosis: that of rapid, efficient execution, and clearance of damaged cells (now apoptotic bodies) so as to prevent

aggravation of inflammation [87, 88]. Removal of apoptotic epithelial cells in the airway (whether by shedding into the lumen or by phagocytosis) may be accompanied soon after by proliferation and/or phenotype shifting of neighboring cells to replace the lost cells. Indeed, both apoptosis and proliferation may be increased in diseases such as asthma [53, 89]. Demonstration of significant numbers of apoptotic cells might not only suggest significant injury and response to injury, but may also suggest a defect in clearance of apoptotic cells (reviewed in [90]), a finding that, for example, may be of importance in emphysema [87]. Accumulation of apoptotic bodies may exert direct inhibitory or damaging effects on neighboring cells and may lead to continued inflammation [15], though this has yet to be specifically demonstrated in airway mucosa.

Therefore, particularly in disease states, the true proportion of apoptotic cells may be underestimated by morphological methods that depend on the continued presence of the dead or dying cell.

Since airway epithelial cells have the CD95 receptor, it follows that ligating that receptor should activate pathways that lead to cell death. Both in primary cells and in cell lines, this is clearly true [20, 50]. However, of interest is not that epithelial cells die in response to CD95 ligation, but that in most culture systems using primary cells collected from normal subjects or in cell lines, maximal activation of this receptor elicits death in only 10–20% of the cells present over a 24 to 48 hr time period. This is in sharp contrast to studies done in lymphocytes, eosinophils, and other inflammatory cells, in which CD95 ligation generally elicits >80% cell death in less than 8 hr (as several of many examples, see [64, 91, 92]). One might ask, if epithelial cells express both ligand and receptor, why one epithelial cell does not kill its neighbor? The answer may be that epithelial cells, at least under normal conditions in the airway (or in standard culture), are relatively resistant to CD95-induced death signaling, either by an inability to cluster and activate the receptor trimer required for FADD activation or further along the signaling pathway. This further suggests two possibilities. The first is that either a second "costimulatory" signal is required for maximal killing. No such signal has been demonstrated in airway epithelium, but signals that sensitize cells to death receptor stimulation are seen in other systems. For example, IFN-γ and TNF-α sensitize human endometrial stromal cells, normally apoptosis resistant, to CD95-mediated cell death due to an upregulation of CD95 expression [93], and interleukin (IL)-10 protects mouse intestinal epithelial cells from Fas-induced apoptosis by downregulating Fas expression and regulating the expression of death domain components [94].

A second possibility is that a change in the environment elicits a reconfiguration of the receptor that makes it more amenable to activation. Data to support such a hypothesis in airways are lacking, but, in other systems, it is clear that CD95 receptor regulation in turn regulates the ability to switch on apoptosis. For example, expressing a mutant CD95 that lacks a death domain, and therefore cannot initiate DISC formation, blocks apoptosis ordinarily elicited with an anti-CD95 antibody, even as the receptor trimers aggregate [95]. Expression of the cellular FLICE-like inhibitory protein (c-FLIP), a component of the death-effector domain, can either inhibit or activate both CD95 and other death receptors dependent on context (reviewed in [24]), but this regulator has yet to be described in airway epithelium.

In the normal airway epithelium then it is clear that apoptosis, like proliferation, is tightly regulated to the point that it is uncommonly seen as best as can be detected with current methods. Epithelial cells can respond to death stimuli in the culture environment but even then there is relative resistance. Understanding the "default" setting of "no death" may help us to understand compensatory responses and disease states in which apoptosis clearly is activated.

6. Apoptosis as a Mechanism to Maintain Epithelial Cell Homeostasis

Both in disease states such as asthma and COPD and after various environmental exposures, significant goblet cell hyperplasia (GCH) may be seen [96–98] in airway mucosa. Cytokines such as IL-13 and IL-4 can stimulate phenotype shifting of large airway basal [99] or small airway epithelial (Clara) cells [100] to mucoid cells by inducing mucin expression [99–104]; two separate reports suggest that ciliated cells may also shift to a mucoid phenotype under certain conditions [105, 106]. The bacterial wall inflammatory factor lipopolysaccharide (LPS) also can induce GCH in cell culture models of epithelial cell phenotype shifting [107, 108] as can irritants such as cigarette smoke [109]. There may also be an increase in the total number of epithelial cells over time with most of the new cells manifesting a mucoid phenotype [110]. Resolution of goblet cell hyperplasia is associated with downregulation of mucin protein expression and either phenotype shifting of these cells to a Clara or serous cell phenotype [100] or absolute reduction of goblet cell numbers by apoptosis [111–113].

The appearance of new goblet cells in GCH may be due to an inhibition of apoptosis. Harris and colleagues [110] demonstrated increased expression of Bcl-2 in mucous cells after LPS instillation in Norway rats; Bcl-2 positive mucous cells decreased to normal levels just prior to resolution of GCH. In their study, stable overexpression of Bcl-2 increased LPS-induced GCH compared to wild-type mice [110]. A similar expression of Bcl-2 and GCH was seen in nasal epithelium after ozone exposure in rats [114]. Resolution of GCH may require more than downregulation of Bcl-2: expression of the proapoptotic mitochondrial regulator Bax, which can heterodimerize with Bcl-2 and block its function [115], is associated with the loss of goblet cells in the resolution phase of GCH, and treatment with agents such as IFN-γ, which increases Bax expression, is associated with increased clearance of goblet cells [116], even as treatment with an anti-Fas antibody fails to clear these cells [113]. Other modulatory pathways such as that coupled to the epidermal growth factor receptor (EGFR) also may modulate GCH induced by either allergen exposure in cultured H292 epithelial cells [117] or Sendai virus exposure in mice [118],

such that blocking EGFR signaling induced apoptosis in goblet cells.

These studies serve as one striking example of how apoptosis may be intimately involved in regulating the phenotype and presence of airway epithelial cells in response to environmental perturbations. Both the appearance and disappearance of goblet cells may require (resp.) inhibition or initiation of apoptosis. Both offer potential therapeutic checkpoints to drive the airway mucosa towards a more homeostatic model in response to chronic illness.

7. Apoptosis in Airway Epithelium in Disease

Epithelial cell apoptosis is a feature found in damaged and inflamed airways. While increased apoptosis is thought to contribute to airway damage and pathogenesis, it may also be a consequence of reparative processes that attempt to remove dead, dying, or damaged cells.

Discussion of epithelial cell apoptosis in airways diseases is hampered by methodological problems. In addition to the issues of detachment of apoptotic cells into the airway lumen and the clearance of apoptotic bodies by professional phagocytic cells, it can be difficult to distinguish apoptotic from necrotic cells [14] in airway and lung biopsies. Further, a measurement of apoptosis at a single time point (e.g., via endobronchial biopsy) may not reflect either the state of damage in that airway at that time point, of the disease state elsewhere in the lung at that time point or of the course of the disease over time. Repeated measurements of epithelial cell apoptosis *in vivo* over time are currently not possible, as there are no specific markers to be found in sputum (noting the recent study of Chimenti et al. [82] that suggests at least the possibility of identifying TUNEL-positive epithelial cells in sputum collected from asthmatic subjects), bronchoalveolar lavage fluid, or exhaled breath condensate, and repeated biopsy of the airways, in addition to methodological problems, has obvious limitations in terms of patient access and risk. The discussion that follows for each disease then is based on limited *in vivo* studies supplemented by mouse and culture models.

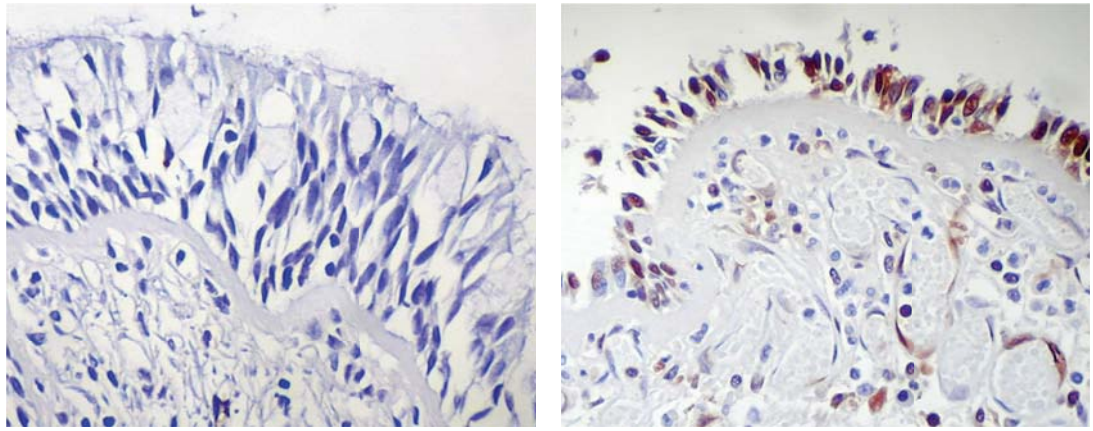
7.1. Asthma and Airway Inflammation. Asthma has been recognized since antiquity. The word derives from the Greek meaning "short of breath." By the end of the 19th century, Henry Hyde Salter had recognized the appearance of an asthmatic airway with inflammation and hypertrophy of smooth muscle [119], and Sir William Osler had described the relation of allergy, hay fever, familial predisposition, childhood onset, the presence of Leyden crystals and Curschmann spirals in sputum, and paroxysms to asthma [120]. In the early to mid 20th century, asthma was seen as a disease of intermittent bronchospasm and treated with various bronchodilators. But from the 1920s, inflammation was recognized as pathogenetic to asthma (for a review see [121]), and, more recently, the concept of "remodeling", a description of a chronically inflamed, narrowed asthmatic airway with smooth muscle hypertrophy, a thickened basement membrane, and—importantly for this discussion—chronic, persistent, focal to widespread epithelial damage along with goblet cell hyperplasia [98]—as an end result of inflammation, has defined the disease process [122, 123].

The epithelium is a target of inflammatory and physical insults in both acute asthmatic inflammation and in chronic asthmatic remodeling [1]. Epithelial injury is common even when the clinical state of asthma is mild [123, 124] and is persistent over time [1]. Epithelial damage correlates with airway hyperreactivity and may be seen in newly diagnosed asthma [125, 126]. Epithelial damage is more than a manifestation of injury: it is an effector of the airway inflammation that marks chronic asthma [127–129]. Epithelial injury leads to disordered regulation of submucosal myofibroblasts and fibrinogenesis [130, 131] and release of chemokines such as IL-8 and eotaxin [132, 133]. Mucosal damage may be extensive in severe or fatal asthma [134] but is more focal in mild-moderate asthma [125, 135].

7.1.1. Human Studies. Epithelial cell apoptosis is difficult to assess in human asthma, both for morphological issues such as sampling bias and the aforementioned risk of underestimating apoptosis in damaged airways, and issues due to the variable nature of the disease and variable methods used to classify patients. Studies of apoptotic markers in endobronchial biopsies collected from asthmatic subjects have shown variable results: for example, Vignola et al. [77] examined TUNEL, Bcl-2, and p53 labeled in a cohort of asthmatics, either untreated or treated with in-haled or oral corticosteroid (CS) agents, and in control subjects. In this study no control subject, and few asthmatic subjects, had any TUNEL-positive cells in the epithelial layer; while p53 was not expressed in any group, subjects with asthma, treated or not, had a higher number of cells labeling for Bcl-2 versus control. In contrast, a study by Cohen et al. [89] examined both apoptosis and proliferation in bronchial biopsies collected from normal subjects and subjects with mild or severe asthma. In this study, there was a greater number of TUNEL-positive cells in biopsies of severe asthmatics compared to controls, along with decreased Bcl-2 expression and increased proliferation as noted by labeling for the marker Ki-67. These two studies may be reconciled on the basis of disease severity, as the Cohen study did not demonstrate an increase in apoptosis in mild asthmatics [89]. Yet another study by Trautmann et al. [53] demonstrated apoptotic epithelial cells in a small cohort of patients with mild, persistent asthma, as demonstrated by TUNEL and Hoechst labeling on endobronchial biopsies. In our own laboratory, apoptotic epithelial cells can be demonstrated clearly in endobronchial biopsies of subjects with chronic, persistent asthma; such labeling is almost never seen in biopsies collected from normal volunteer subjects (Figure 3).

Therefore, as best can be demonstrated to date, there are relatively few apoptotic cells present in the airway epithelium of mild asthmatics; more significant apoptosis and proliferation are seen in asthma and may be related to both chronicity and severity.

7.1.2. Effect of Corticosteroids. Corticosteroid therapy in asthma does not necessarily reverse epithelial damage seen in



(a) (b)

Figure 3: Demonstration of apoptotic airway epithelial cells, as demonstrated by TUNEL stain for single-strand DNA nicking using a peroxidase method (brown, with hematoxylin counter label), in an endobronchial biopsy of a normal subject (a) and of a subject with chronic, persistent asthma (b). Note the substantial difference in the height of the epithelium and the gaps in the epithelium at the basement membrane between the two biopsies, and the loss of ciliated cells in asthmatic biopsy. The appropriate controls (not shown here) do not label. Original magnification, 200x. From the author's laboratory.

asthma. Corticosteroid treatment clearly suppresses inflammation in a large majority of asthmatic patients [136–139] and in mouse asthma models of allergen-induced airway inflammation [137, 140], though it is clear that corticosteroids do not alter the natural history of asthma [139]. In addition to the effects on the number and function of inflammatory cells that ordinarily infiltrate the airway mucosa in chronic asthma, CS treatment also inhibits the release of inflammatory mediators secreted by epithelial cells such as RANTES [141], GM-CSF [142], and eotaxin [143].

However, the role of corticosteroids on epithelial cell survival and death is less clear. That some asthmatic subjects have improved airway mucosal integrity and epithelial cell anatomy following treatment with inhaled CS is unequivocal: for some subjects, clinical improvement is accompanied by evidence of restoration of normal epithelial anatomy as demonstrated on endobronchial biopsies [144]. Further, CS treatment of cultured epithelial cells may inhibit cell death induced by cytokines such as IFN-γ or TGF-β. Dexamethasone at concentrations of 1 mM inhibits IFN-γ induced cell death in the A549 peripheral lung adenocarcinoma cell line, perhaps by inducing expression of hIAP [145]. Contradictory reports suggest that TGF-β can induce epithelial cell apoptosis that is blocked by concurrent treatment with budesonide [146] or can prevent epithelial cell apoptosis that is induced by dexamethasone [147].

Balancing these studies are reports that CS treatment of cultured human airway epithelial cells and cell lines may induce apoptosis [50, 147–149]. Either dexamethasone or budesonide in concentrations of 1–10 μM in culture, a dose that when adjusted for airway surface area and sol volume calculates to the high end of the point concentration of an inhaled corticosteroid on epithelium *in vivo*, elicits caspase-mediated apoptosis that requires disruption of mitochondrial polarity and release of cytochrome c [50]. Overexpressing either Bcl-2 or Bcl-xL inhibits CS-induced apoptosis in this model. A follow-up study demonstrated that dexamethasone treatment in Balb-c mice for 3 days to 4 weeks also elicited increased epithelial cell apoptosis, as measured by TUNEL labeling and labeling for the 85 kD fragment of polyadenine ribopolymerase (PARP), a nuclear enzyme cleaved early in apoptosis [75]. One additional study also suggests that concurrent treatment of cultured human airway epithelial cells and cell lines with the beta-adrenergic agonists albuterol or formoterol can block CS-induced apoptosis [149]—this may provide one potential explanation as to why relatively few apoptotic epithelial cells are seen in the airways of asthmatic subjects, as most of these subjects receive such agents as part of their antiasthma controller therapy.

7.1.3. Allergen Exposure, Asthma, and Epithelial Cell Apoptosis. Mouse models of airway inflammation induced by exposure to allergen are a time-honored, useful model to explore the effects of an allergen on airway structure and function and, unlike in human studies, permit careful assessment of mechanism and function. The classic model involves the sensitization and challenge to ovalbumin (OVA) [150], followed by collection of tissue, BAL fluid, or other samples at key time points following challenge. Genetic manipulation of the mouse, adoptive transfer of selected lymphocytes, or other treatments can modify airway inflammation. From these many studies, a clear picture of murine airway inflammation has developed that has informed our understanding of human airway inflammation in asthma.

Two laboratories have demonstrated increased epithelial cell apoptosis after OVA challenge in mice. Truong-Tran et al. [151] examined apoptosis markers such as caspase-3 activity in an OVA model in Balb/c mice. Allergen challenge elicited airway inflammation and airway hyperresponsiveness, as expected, and also an increased number of apoptotic bodies in the airway mucosa and increased immunolabeling for

activated caspase-3; both were increased by concurrent dietary depletion of zinc, a factor that the authors suggest may be protective against epithelial damage.

A second study from our laboratory [75] examined epithelial cell apoptosis after OVA challenge with and without concurrent corticosteroid therapy. This study grew from an earlier observation (discussed above) that corticosteroid treatment could induce apoptosis in cultured primary human airway epithelial cells and cell lines [50]. We hypothesized that corticosteroids could cause cell death of airway epithelium *in vivo*, and the resulting loss of epithelial cells might explain in part the damage to and denudation of the airway mucosa in chronic asthma, despite control of other markers of inflammation. To test this, Balb/c mice were sensitized and challenged to OVA, and both sensitized mice and control, unsensitized mice were treated at selected time points with dexamethasone in doses calculated to be at the high end of a therapeutic regimen for asthma. Allergen challenge also elicited increased TUNEL and p85-PARP labeling over the first 14 days after challenge, and this was not decreased by concurrent corticosteroid treatment.

These two papers have made clear the association of epithelial cell apoptosis and allergen-induced airway inflammation. Human studies to examine the association and to perhaps demonstrate causality of allergen challenge on epithelial cell apoptosis, however, have not been done to date. One paper by Robertson et al. [69] examined the expression of the TNF family mediator TRAIL, which can induce apoptosis and its receptors DcR2, DR4, and DR5 following segmental allergen challenge by bronchoscopy in a cohort of asthmatic and nonasthmatic subjects with ragweed allergy. In asthmatic subjects, instillation of ragweed into the airways elicited increased epithelial TRAIL labeling within 2 days along with decreased immunostaining of DR4 and DR5 in eosinophils and macrophages. However, no labeling of endobronchial biopsies to examine epithelial cell apoptosis was done.

7.1.4. Airway Epithelial Cell Damage and Apoptosis in Response to Exercise and to Cold/Dry Air. A substantial proportion of asthmatic patients demonstrate worsening symptoms and airway inflammation as a result of exposure to cold, dehumidified air associated with exercise [152–154]. Cold air challenge and hyperpnea both are clinical and laboratory models used to induce bronchoconstriction in susceptible subjects with airway hyperreactivity [155, 156]. Athletes, even those not suspected of having asthma, may also have airway inflammation in response to cold air exposure [157–159]. Even in more moderate environments such as swimming or rowing in which there is substantial hyperpnea, elite athletes may have inflammatory cells present in induced sputum and in airway lavage [159, 160]. Airway epithelial damage in athletes may be seen: for example, in a cohort of nonasthmatic long-distance runners, increased numbers of shed bronchial epithelial cells and of TUNEL-positive apoptotic epithelial cells were seen in induced sputum after a half-marathon race [82]. These findings have been explored in a mechanistic model in mice, in which lung sections collected from sedentary and endurance-trained mice were examined [161]. Compared to sedentary mice, the bronchiolar epithelium of endurance-trained mice demonstrated a progressive loss of ciliated cells and both increased apoptosis and increased proliferation, along with infiltration of CD45+ leukocytes into the mucosa. These changes may represent an adaptive response to increased ventilation during exercise.

In summary, asthma, asthma treatment, and Th2-mediated airway inflammation are associated with central airway epithelial cell apoptosis. The significance of this cell death is not yet clear, nor is it clear whether epithelial cell apoptosis represents a compensatory, adaptive response to clear damaged or dying cells or represents a maladaptive, pathologic response that over time contributes to the epithelial injury and airways remodeling process that characterizes chronic asthma.

7.2. Viral and Bacterial Infection. Viral infection of the airways is increasingly demonstrated as a leading cause of exacerbations of clinical asthma, particularly in childhood [162]. Infections with viruses such as adenovirus (AdV), rhinovirus (RV), respiratory syncytial virus (RSV), influenza virus, and parainfluenza virus (PIV) occur early in the lower respiratory tract, are association with exacerbations and hospitalizations for asthma [163, 164], and are recognized as an important risk factor in early childhood for the development of persistent asthma later in life [165, 166]. Patients with asthma with frequent viral-induced exacerbations have worse pulmonary function and a worse clinical course than patients without such frequent infection [167], a finding that may first develop in infancy [168]. Frequent viral infection in infancy may alter Th2 responses [169], particularly when combined with allergen exposure [170]. The airway epithelium is a central target of viral infection and replication, and epithelial cell damage, release of cytokines, and other factors. Apoptosis then can be seen as a central part of host defense; viruses may use apoptosis to subvert host defense for their own survival. One broad method for doing so is for a virus to provoke apoptosis of the infected cell. The potential advantage of this is that it may facilitate viral egress after replication and thus spreading and survival of the virus (reviewed in [171]).

Infection with RSV can induce apoptosis in epithelial cells. This was clearly seen in the study of Kotelkin and colleagues [172] in which RSV infection of cultured primary tracheal or small airway epithelial cells, or cell lines, elicited apoptosis that was associated with both caspase-8 (receptor mediated type I) and caspase-9 (mitochondrial associated type II) activation and with expression of both pro- and antiapoptotic Bcl-2 family members. Interestingly, RSV also induced TRAIL expression and expression of both the DR4 and DR5 receptors, not seen in normal, uninfected cells. A more recent study demonstrated that RSV infection was associated with recovery of soluble TRAIL in airway lavage fluid and the expression of the TRAIL receptors R1 and R2 in the airway epithelium of children with RSV infection and mechanical ventilation [72]. These findings suggest that TRAIL may contribute to epithelial injury during severe RSV

infection. A third study examined the apoptosis-inducing effect of RSV on primary nasal, tracheal, and bronchial epithelial cells grown in culture. In this study, RSV elicited apoptosis that was increased after knockdown of endogenous nerve growth factor [173].

Adenoviral infection also can induce apoptosis in epithelial cells. In a guinea pig model of AdV infection, Singhera and colleagues [71] demonstrated increased, time-dependent apoptosis, as measured by detection of the p85 fragment of PARP, along with expression of the DR4 and DR5 receptors. Interestingly, corticosteroid treatment delayed apoptosis in cultured epithelial cells following AdV infection and allowed increased viral particle production. In a chronic infection model, infection plus sensitization and challenge with ovalbumin led to increased numbers of apoptotic epithelial cells; again, corticosteroid treatment decreased the number of apoptotic cells. These experiments suggest that corticosteroid use may actually contribute to decreased viral clearance and thus prolonged infection.

Another virus potentially important to asthma is RV. While RV infection is less toxic to epithelial cells in different models, it can induce apoptosis via activation of caspase-9 in cultured, nonpolarized epithelial cells in culture [174]. However, apoptosis was not seen in RV-infected, polarized, differentiated epithelial cells, even as RV infection disrupted the epithelial barrier function as measured by transepithelial resistance [175].

Influenza A infection elicits an inflammatory airway response with epithelial cell secretion of cytokines and chemokines. One study has examined influenza-A-infection-induced apoptosis in the H292 human mucoepidermoid bronchiolar carcinoma cell line. In this study, Brydon et al. [176] demonstrated that inhibiting apoptosis using caspase inhibitors increased chemokine secretion, suggesting that the observed inflammatory response in influenza would be greater if cell death did not occur. Another study demonstrated that, in H292 cells, influenza infection elicited apoptosis by upregulating mitogen-activated protein kinase signaling pathways that include c-Jun and p38-MAPK [177]. The avian H9N2 influenza virus elicits apoptosis in primary, polarized, differentiated airway epithelial cells with activation of caspase-9 and release of mitochondrial cytochrome c [178]. In this study, IFN-β was antiviral and antiapoptotic, which fits the known role of interferons in blocking viral infection [171]. In this light, a study by Chan et al. demonstrated that more differentiated airway epithelial cells in culture were more resistant to H5N1 influenza infection [179]; the authors noted that an undifferentiated epithelium, as might be seen following damage due to virus or other agents, could be easily infected. Examining apoptosis in both undifferentiated or growing epithelial cells versus a tightly organized differentiated epithelium with a highly polarized and tight barrier may be required to understand how influenza and other viruses use cell death to their advantage in clinical infection.

Viral infection also may suppress apoptosis so as to ensure their replication and survival by preventing the death of the cell. While cytomegalovirus and certain herpesviruses are known to inhibit apoptosis (reviewed in [171]), no data suggest that such suppression occurs after viral infection of airway epithelium.

Taken together, these studies suggest that infection with viruses that commonly cause respiratory disease such as RSV, AdV, and influenza elicit apoptosis of airway epithelium. The interplay between epithelial damage and loss, inflammation, and viral replication is not yet clear from these studies. Studies are needed to demonstrate whether blocking apoptosis in viral infection leads to clear changes in the inflammatory state and epithelial damage, and better studies in human subjects, particularly children, are needed to demonstrate the association of epithelial cell damage, loss, and apoptosis with ongoing airway inflammation and loss of asthma control. Though challenging, such studies will provide important answers as to whether inhibiting apoptosis in the setting of viral infection leads to better, or worse, clinical outcomes.

7.3. Cystic Fibrosis. Cystic fibrosis (CF) is a multisystem, genetic disease caused by mutations in the gene encoding CFTR, which leads to decreased chloride secretion and increased sodium absorption, with resulting decreases in lumenal liquid, in airways, the pancreas, and other organs lined by epithelial cells (reviewed in [180]). In airways, this leads to dehydration of airway mucous with resulting poor mucous clearance, infection, particularly with organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, inflammation, and, over time, bronchiectasis and fibrosis of the airway [180, 181].

Clearly the epithelium is central to disease pathogenesis in CF, and airway inflammation along with damage to and loss of epithelial cells are prominent in established bronchiectasis and airway fibrosis in this disease [182]. Over the past decade, apoptosis as one of several mechanisms for airway epithelial cell loss in CF airways has become recognized.

Expression of CD95 and CD95L, along with markers of apoptosis such as DNA fragmentation, was first reported by Durieu et al. [52] in a study of surgical lung lobectomies obtained from a small cohort of CF patients. In this study, CD95L expression was markedly increased in bronchial epithelial cells, and TUNEL labeling demonstrated apoptotic cells both in the mucosa and in submucosal gland epithelial cells. Harris et al. [110] demonstrated increased Bcl-2 presence in airway epithelium in lung blocks collected from patients with CF compared to normal airways; this was most prominent in goblet epithelial cells. Bcl-2 expression in this setting may be a general, compensatory response to permit survival or may permit preferential development of goblet cells that then would secrete increased mucous into the CF airway.

Apoptosis of CF-derived epithelial cells in culture also has been demonstrated. Ligating CD95 induces apoptosis (as expected) in normal tracheal epithelial cell lines but increased apoptosis in the CF-derived cell line CFT-2 [183]. The *Pseudomonas aeruginosa* strain PAO1 induces apoptosis, as demonstrated by TUNEL labeling, in about 10% of primary epithelial cells over 8 hr of exposure but 50% in the 9HTEo-transformed cell line that lacks tight junctions [184]; treating another transformed cell line, 16HBE14o-

that has high-quality tight junctions, with EGTA made them more susceptible to PAO1-induced apoptosis. In contrast, stable transfection of a constitutively active variant of the regulatory R-domain of CFTR did not alter rates of apoptosis elicited by PAO1, nor did PAO1 elicit significant apoptosis in *cftr* $^{-/-}$ mice [184]. These data suggest that the intrinsic loss of CFTR is less important in epithelial cell survival than morphologic changes such as barrier integrity. This conclusion is challenged by a more recent study examining apoptosis after airborne particulate matter (PM) exposure in two epithelial cell lines, IB3-1 that contains compound heterozygote delta F508 and W1282X nonsense CFTR mutations, and S9 that is derived from the IB3-1 cell line with the CF phenotype corrected by transfection with wild-type adenoassociated viral CFTR. Upon exposure to PM in a standard model for 1 hr, IB3-1 cells had a rate of apoptosis greater than six fold higher than that seen in S9 cells; this was associated with disruption of mitochondrial polarity and activation of caspase-9 and blocked by overexpression of Bcl-xL [185]. Increased susceptibility to oxidative stress may account for the effect of PM in this model, and oxidative-stress, a known inducer of apoptosis in other systems, may also have other effects that induce cell death in CF airway epithelium. Ahmad and colleagues [186] examined the expression and activity of sarcoendoplasmic reticulum calcium ATPase (SERCA), a regulator of calcium homeostasis, in normal and CF epithelium and tissue. SERCA2 expression was decreased both in airway biopsies and in differentiated, polarized cultured epithelial cells from CF subjects and in CF epithelial cell lines, and expression of the Δ508-mutated CFTR in cell lines led to decreased SERCA2 expression. SERCA2 displaces Bcl-2 from the endoplasmic reticulum [187], and silencing of the SERCA2 gene enhanced epithelial cell death due to oxidative stress and to treatment with TNF-α [186].

Pseudomonas infection may also provoke epithelial cell apoptosis by inducing the expression of cationic innate host defense peptides that injure the host. One such cationic peptide is LL-37, the predominant cleavage product of human cationic antimicrobial peptide (hCAP)-18, the human cathelicidin (reviewed in [188]). LL-37 is secreted by both neutrophils and epithelial cells and is upregulated in response to infection and inflammation [188, 189]. Expressing LL-37 in murine lung enhances the clearance of pulmonary *Pseudomonas aeruginosa* [190]. LL-37 can induce apoptosis in both primary cells and in cell lines [191–193] and does so via activation of the intrinsic pathway with release of cytochrome c and activation of caspase-9 [193]. Interestingly, in the latter study, cell death required the presence of both the cationic protein and live bacteria, and either alone was incapable of inducing apoptosis [193]. The effect of other cationic proteins such as the eosinophilic cations or that of synthetic cationic proteins such as polylysine have not been examined in the context of airway epithelial cell death.

Taken together, these studies show that epithelial cell apoptosis may be present in CF airways and that CF epithelial cells are perhaps more susceptible to apoptotic cell death following oxidant stress, bacterial infection, and perhaps to host defense factors (LL-37 as one demonstrated example). The relative contribution of apoptosis to epithelial damage in CF airways, as well as the contribution overall to CF pathophysiology, is yet to be defined.

7.4. COPD. Chronic obstructive pulmonary disease (COPD) arises as a result of the inhalation of noxious stimuli to the lungs, most commonly cigarette smoke. Damage to the central airways (chronic bronchitis) and to the peripheral lung (emphysema) commonly occurs. Several mechanisms contribute to the pathogenesis of COPD, including influx of inflammatory cells into the lung, disruption of the balance between proteolytic and antiproteolytic activity, and oxidative stress [194].

Some recent studies have suggested increased levels of apoptosis in peripheral lung endothelial cells [195, 196] and alveolar inflammatory cells [197, 198] (also reviewed in [199]). It is surprising, given the levels of oxidative stress and the toxins inhaled in cigarette smoke, that apoptosis has not been investigated rigorously in central airway epithelial cells. One recent study from Korfei et al. [200] examined markers of apoptosis in explant tissue obtained from a small cohort of patients with COPD, idiopathic pulmonary fibrosis, or donated lung and demonstrated no evidence of such markers in COPD. Another study examined the presence of apoptosis in central airway epithelial cells collected by endobronchial brushing of 2–4 mm airways from 20 normal subjects and 23 subjects with COPD [201]. Several assays to assess apoptosis were done to minimize the risk of technical artifact skewing the results. Small airway epithelial cells collected by brushing from the patients with COPD had increased labeling for the presence of apoptosis by each assay; this was not affected by either the age of the subject or by smoking status. A recent study in which mice were exposed either acutely or chronically to ozone demonstrated that chronic exposure led to both central and peripheral airway epithelial cell apoptosis as measured by immunoreactivity to caspase-3 and the apoptosis protease activating factor-1 (APA-1) [202]. How apoptosis in the more central airways relates to the genesis of chronic bronchitis or airway inflammation is not yet known.

Peripheral alveolar epithelial cells in smoking subjects with COPD also show evidence of apoptosis markers such as p53 [203] or TUNEL [198, 204]. It is interesting to speculate that such cell death may contribute to the pathogenesis of emphysema, but no firm data has emerged.

7.5. Interstitial Lung Disease. Interstitial lung diseases comprise a spectrum of illnesses that have at their core fibrotic damage to the peripheral lung. One of these, idiopathic pulmonary fibrosis (IPF), is a progressive disease in humans that has, as central to its pathogenesis, injury to small airways and alveolar epithelial cells [205, 206]. Over time continued injury and reaction to injury lead to impaired epithelial repair, phenotype shifting of fibroblasts to myofibroblasts, matrix deposition, and scarring. Several types of injuring agents such as oxidative stress have been postulated as causes of the initial injury [207], even though antioxidant therapy has but modest benefit in these patients [208, 209].

Apoptosis is clearly recognized in the alveolar epithelium in both human tissue samples of patients with IPF [210, 211] and in mouse models of lung fibrosis [212, 213]. Increased expression of Fas [214–216] and of apoptosis signaling pathway intermediates such as p53 [210, 216, 217] are seen which may help explain the loss of epithelial cells. In contrast, damage to and apoptosis of more central airway epithelial cells are not seen [211].

Central airway epithelial cell apoptosis is not a prominent feature of other interstitial lung diseases, such as sarcoidosis or the collagen-vascular-associated lung diseases, as reported to date.

7.6. Lung Transplantation. Lung transplantation (LT) remains the best hope for selected patients with end-stage lung diseases. Chronic allograft rejection, clinically manifested as bronchiolitis obliterans syndrome (BOS) and pathologically as obliterative bronchiolitis (OB), remains a major limitation to long-term survival: BOS occurs in 40–60% of lung transplant recipients within 4 years [218, 219] and is the leading cause of death after the first year [220, 221]. Allograft rejection is mediated mainly by recipient alloreactive CD4+ and CD8+ effector T lymphocytes [222, 223] developed against mismatches in HLA class I and II antigens of the epithelial cells in the allograft [224–226]; these in turn may be suppressed by regulatory T suppressor lymphocytes that promote tolerance [227, 228].

There are few reports of epithelial cell apoptosis in the transplanted human lung. An early study suggested that in transplanted lungs in which obliterative bronchiolitis was present, there were increased numbers of TUNEL-positive cells in large airway, small airway, and alveolar epithelial cells, whereas such labeling was virtually absent in transplant lung specimens that had no evidence for OB [229]. An elegant study of ischemia reperfusion in the transplanted lung collected samples from peripheral donor lungs during cold ischemia, warm ischemia, or after graft reperfusion at the time of implantation. In this study, TUNEL labeling demonstrated significant alveolar but not more central epithelial cell apoptosis during the time of reperfusion but not during either ischemia periods [230].

Animal lung transplantation models provide important data concerning the role of apoptosis in obliterative bronchiolitis and in ischemia-reperfusion injury. Increased numbers of apoptotic epithelial cells are seen prior to sloughing and obliterative bronchiolitis in the mouse heterotopic tracheal transplant model [231] and in a similar model in swine [232], though the degree of apoptosis is modest compared to the subsequent loss of epithelial cells, suggesting that other processes such as necrosis or autophagy are as or more important. A more recent study examines epithelial cell apoptosis in an orthotopic mouse tracheal transplant model with preservation of ventilation through the transplanted airway [233]. In this study, ventilation was permitted or occluded through the transplanted trachea, and morphometric and immunohistochemical measurements were made 28 days after transplant. Epithelial cell morphology was better preserved in the ventilated group with more numerous ciliated cells, and TUNEL-labeling was substantially less in these allografts, ~12%, versus those that were not ventilated, ~66%. Evidence for obliterative airways disease was present in the nonventilated allografts suggesting an association between OB and epithelial cell apoptosis. Other studies suggest that airway obliteration characteristic of the heterotopic tracheal allograft model does not occur in orthotopic allografts and that recipient epithelial cells migrating into the donor graft may actually prevent obliteration [234–236]. The role of apoptosis in this process is not known.

One intriguing study examined the role of treatment with an endothelin-A/B receptor antagonist, SB209670, to block apoptosis following ischemia-reperfusion injury in a dog model of lung allotransplantation [237]. Blocking the endothelin receptors led to lower numbers of apoptotic epithelial cells in the transplanted peripheral airways and alveoli as assessed by TUNEL labeling. This may be due to the ability of this antagonist to increase Bcl-2 expression [238].

One paper has examined epithelial cell apoptosis in a culture model using the KCC-266 cell line; treatment with an antibody that activates class I HLA responses (W6/32) elicits both proliferation and apoptosis over 48 to 72 hr [225]. However, the cell line is not characterized or described in this study or elsewhere, and it is not clear whether this is a central or alveolar-derived cell.

7.6.1. Environmental Exposures. Environmental agents may disrupt the integrity and function of the airway epithelium. In addition to allergen exposure as noted above, various gasses and particles may elicit damage via initiation of apoptosis. One group of agents include the incompletely-combusted components of fossil fuels such as polycyclic aromatic hydrocarbons (PAHs). One such family of hydrocarbons, benzo[a]pyrenees, can elicit apoptosis in primary small airway epithelial cells, but not the peripheral A549 adenocarcinoma epithelial cell line, in culture [239], by increasing free cytosolic calcium levels [240].

Fine particulate matter also can induce apoptosis in CF-derived epithelial cells as previously noted [185]. Another study of the effect of fine particulate matter (PM2.5) on the L132 human lung epithelial cell line demonstrated that exposure-induced apoptosis by both receptor-mediated (caspase-8 activation) and mitochondrial (cytochrome c release and caspase-9 activation) pathways [241]. These fine particles are composed of a variety of inorganic and organic chemicals, including PAHs, and it is not clear which part of the particle is responsible. To make this more complicated, another study demonstrated that a water-soluble fraction of PM2.5 actually inhibits apoptosis induced in three different epithelial cell lines and in primary epithelial cells [242]; the authors suggest that blocking apoptosis may contribute to prolonged inflammation and impaired repair in airways after pollution exposure. In support of this, Rumelhard et al. [243] examined the expression and role of EGFR ligands such as amphiregulin, heparin-binding epidermal growth factor-like growth factor (HB-EGF), and TGF-α in primary airway epithelial cells and the 16HBE14o- cell line after exposure to PM2.5. Secretion of each ligand was seen, and each elicited expression of cytokines such

as granulocyte-macrophage colony stimulating factor (GM-CSF). This study was subsequently replicated in primary airway epithelial cells using PM2.5 with different PAH and metal contents, demonstrating significant overlap between the groups suggesting that the exact composition of the particulate matter is less important [244]. Clearly, the effect of environmental agents on epithelial cell apoptosis, as well as the subsequent effect upon airway homeostasis, requires further investigation.

One interesting paper notes the effect of mechlorethamine, a functional analogue of vesicant sulfur mustard used in chemical warfare, on differentiated HBE1 cells in culture. In this model, mechlorethamine treatment elicited epithelial layer disruption with evidence of both cytotoxic and apoptotic changes in a concentration and time-dependent manner [245].

8. Conclusions

Apoptosis is an important regulator of epithelial cell survival in several important clinical diseases. In contrast to inflammatory cells which undergo apoptosis readily and quickly after an appropriate stimulus, epithelial cells have some innate resistance to similar provocation. Understanding this relative resistance to cell death may hold a vital clue for understanding the role of apoptosis in airway homeostasis and may also have important implications for diseases that are epithelial based but not touched on in this paper, such as lung cancer. While this paper has not considered the roles of necrosis and autophagy in epithelial cell death, the former especially may be as, or more, important in selected airway conditions. Nevertheless, apoptosis clearly is involved in epithelial cell death in several inflammatory airways diseases.

There is regional variation in the response to apoptotic stimuli: when directly assessed, small airways (bronchiolar) epithelial cells are more sensitive than those collected from larger bronchi or trachea. There is significant variation in the response to activation of death receptors and in receptor-mediated versus stress-activated (via mitochondrial polarity disruption) apoptosis. Again, the reasons for this are not understood.

Finally, apoptosis is required to return an inflamed airway, or one that is hyperplastic or metaplastic, to normalcy. The controlling mechanisms by which this occurs are still not understood. Recognizing when and how apoptosis is important to airway inflammation and how to turn that process to our advantage offers potential therapeutic targets for several airways diseases.

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Review Article

Responses of Airway Epithelium to Environmental Injury: Role in the Induction Phase of Childhood Asthma

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The pathogenesis of allergic asthma in childhood remains poorly understood. Environmental factors which appear to contribute to allergic sensitisation, with development of a Th2-biased immunological response in genetically predisposed individuals, include wheezing lower respiratory viral infections in early life and exposure to airborne environmental pollutants. These may activate pattern recognition receptors and/or cause oxidant injury to airway epithelial cells (AECs). In turn, this may promote Th2 polarisation via a "final common pathway" involving interaction between AEC, dendritic cells, and CD4+ T lymphocytes. Potentially important cytokines produced by AEC include thymic stromal lymphopoietin and interleukin-25. Their role is supported by in vitro studies using human AEC, as well as by experiments in animal models. To date, however, few investigations have employed models of the induction phase of childhood asthma. Further research may help to identify interventions that could reduce the risk of allergic asthma.

1. Introduction

Asthma is one of the most common chronic diseases affecting children, especially in economically developed nations. For example, in Australia the prevalence of current asthma in children aged 0–15 years is approximately 11% [1]. Childhood asthma is strongly linked to atopy, which in turn is characteristically associated with a Th2-biased immunological response [2–4]. While this relationship is well documented, the pathogenesis of childhood asthma remains largely unexplained.

However, it is clear that both genetic predisposition and a variety of environmental factors contribute to the development of allergic asthma [4]. Notable among the environmental factors that appear to be crucial in the induction of disease is respiratory viral infection, in particular with rhinovirus (RV) or respiratory syncytial virus (RSV). The association between childhood infections and asthma is complex, because at least in some settings, repeated early-life exposure to infectious agents may reduce the likelihood of developing allergic diseases [5]. Despite this, epidemiological studies strongly suggest that lower respiratory viral infections associated with wheezing, occurring within a critical period of development in early childhood, play an important role in the subsequent development of asthma in children who are repeatedly exposed to inhaled allergens [6–10].

Another clearly defined risk factor for childhood allergic asthma is early-life exposure to airborne environmental irritants. The importance of exposure to environmental tobacco smoke is well established [11, 12]. More recently, a number of large population-based studies, including prospective cohort studies, have clearly defined the increased risk of development of asthma in children exposed to traffic-related particulate pollutants [13–15]. The adverse respiratory effects of such pollutants, especially diesel exhaust particulates (DEPs),

are now recognised as a significant public health problem [16].

Somewhat more contentious is the association between the use of paracetamol (acetaminophen) in infancy or childhood and the subsequent development of asthma [17, 18]. The evidence for an increased risk of asthma following exposures to other environmental chemicals is much less convincing [19].

Fundamental questions remain unanswered about the underlying mechanisms by which environmental factors promote the development of childhood asthma. In particular, if atopy and a Th2-biased immunological response are indeed precursors to the development of childhood asthma, a key issue is how does injury by environmental factors drive an allergic response?

A possible "final common pathway," for which there is now growing support, is based on the interaction between airway epithelial cells, dendritic cells, and CD4+ T-lymphocytes.

2. Driving a Th2-Biased Immunological Response

Dendritic cells (DCs) have long been recognised as playing a crucial role in the induction of Th2 polarisation during an immunological response [20]. As is increasingly being understood, the development of allergic immunological responses may be determined by innate host defence responses after initial exposure to pathogens, allergens, or other irritants [21–23]. These lead to local generation of cytokines that stimulate DC, with effects including upregulation of the expression of costimulatory molecules such as CD40, CD80, CD86, Jagged-1, and OX40L, as well as the production of various chemokines (Figure 1) [24–27]. Such molecules collectively function as "instructive" signals that drive initial Th2 polarisation. The subsequent maintenance of the Th2 bias of the CD4+ T cells may be dependent on epigenetic changes [28, 29], which is now a focus of considerable interest in the study of the pathogenesis of asthma [30, 31].

Key factors that may activate and/or drive the maturation of conventional or myeloid DC, to promote Th2-biased differentiation of CD4+ T-lymphocytes, include the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), thymic stromal lymphopoietin (TSLP), interleukin (IL)-25 and IL-33 [32]. Because airway epithelial cells (AEC) can secrete GM-CSF, TSLP, IL-25, and IL-33 in response to injury, as well as chemoattractants for DC such as CCL20, the airway epithelium appears likely to play a critical role in promoting recruitment/survival of DC and Th2 polarisation of the immune response (Figure 1) [33–35].

2.1. Role of TSLP. Accumulating evidence indicates that TSLP activates DC to prime CD4+ T cells for inflammatory Th2 differentiation [36]. In the context of the induction of childhood asthma, a role for AEC-derived TSLP in the induction phase is strongly supported by in vitro studies. Following exposure to DEP in vitro, human AEC generated reactive oxygen species (ROS) and secreted TSLP, which caused DC precursors to enhance expression of OX40L and Jagged-1, which in turn promoted upregulation of Th2 responses [37, 38]. Similarly, AEC exposed to double-stranded RNA or infected with RV or RSV in vitro exhibited marked upregulation of expression of TSLP [39, 40]. In vivo, transgenic overexpression of TSLP in the lungs promoted an antigen-driven allergic inflammatory response [41]. Injury by proteases has also been shown to elicit generation of ROS by epithelial cells, leading to oxidation of lipids, signalling through Toll-like receptor (TLR) 4, and the production of TSLP that drives Th2 responses following subcutaneous immunisation [42]. Because many allergens exhibit endogenous protease activity, it is therefore also possible that inhaled allergens might themselves contribute to enhanced expression of TSLP by AEC.

To date, however, there are no reported in vitro studies using AEC isolated from children nor have there been studies on the role of TSLP in an animal model of childhood asthma.

Further support for a role for TSLP in the development of asthma comes from studies associating single nucleotide polymorphisms in the *TSLP* gene or its promoter region with an increased risk of developing asthma in childhood [43, 44].

2.2. Role of IL-25. Epithelial cell-derived IL-25 is increasingly recognised as being important in the induction of allergic inflammation [45]. TSLP-activated DC induce strong upregulation of the receptor for IL-25 on Th2 cells, thus linking these two cytokine pathways [46]. Recent studies have identified novel populations of cells involved in the innate host response, which contribute to maintaining and enhancing the Th2-biased response by secreting cytokines such as IL-5 and IL-13 in response to IL-25 [47].

We have shown that IL-25 produced by AEC plays a key role in the induction of a Th2-biased inflammatory response following respiratory viral infection [35]. Furthermore, we have recently provided convincing evidence that IL-25 is of crucial importance in the induction phase of childhood asthma. For the latter studies, we used a novel animal model of neonatal infection with pneumonia virus of mice (PVM), a species-specific paramyxovirus which simulates RSV infection in human infants [48]. Following subsequent intranasal sensitisation with ovalbumin and long-term low-level challenge, these mice developed inflammation and remodelling typical of chronic asthma, together with a Th2-biased immunological response [49]. By themselves, neither infection nor allergen exposure led to the development of an asthmatic phenotype. This model therefore simulates the interaction between early childhood infection with RSV and sensitisation to inhaled allergens during the development of childhood asthma.

In this model, we found that there was significant upregulation of expression of IL-25 following neonatal infection with PVM. Therefore, we tested the effects of administration of a neutralising antibody to IL-25 on the development of the asthmatic phenotype, in comparison to administration of an antibody to IL-4, which has long been recognised as having a crucial role in the induction of Th2 responses [50]. Anti-IL-25, administered either during chronic challenge or in

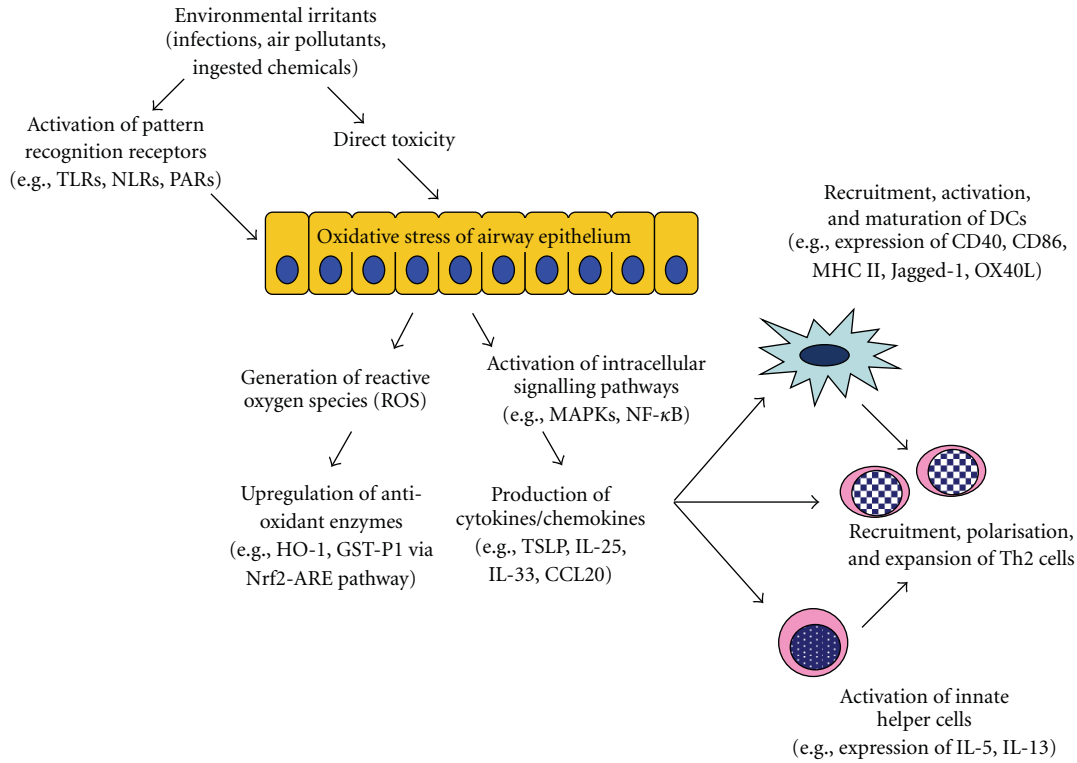

Figure 1: Environmental irritants may activate various pattern recognition receptors on AEC or may be directly toxic to the cells. Exposure to such irritants frequently causes oxidant injury to AEC, leading to generation of reactive oxygen species and upregulation of antioxidant enzyme systems. In parallel, intracellular signalling pathways are activated, triggering production of cytokines that can recruit and stimulate DC, upregulate their expression of costimulatory molecules and promote Th2 polarisation of the CD4+ T-cell response. These cytokines may also activate various populations of innate helper cells, leading to expansion of the Th2 cell population and helping to drive the allergic inflammatory response.

early life alone, prevented key changes of airway remodelling such as subepithelial fibrosis and epithelial hypertrophy, and suppressed development of a Th2 response. Anti-IL-4 was more effective in inhibiting allergic inflammation, prevented goblet cell change but not other features of remodelling, and also suppressed development of a Th2 response [51]. These novel findings suggest that blocking induction of a Th2 response during the neonatal period or later in childhood could be effective for primary prevention of asthma, and that IL-25 might play a crucial role in this process.

2.3. Role of Other Factors. GM-CSF clearly plays a key role in the development of DC [52] and differentiated human respiratory epithelial cells release significant amounts of GM-CSF when exposed to DEP in vitro [53]. However, there is currently no direct evidence of a role for GM-CSF produced by AEC during the induction phase of childhood asthma.

There has been much speculation about the possible contribution of IL-33 to the development of asthma, given the capacity of this cytokine to promote DC maturation towards a Th2-inducing phenotype in vitro [54]. While IL-33 is expressed by airway epithelium in asthmatics [55], there is once again a paucity of direct evidence for a role in the induction phase of allergic asthma.

3. Triggering Cytokine Release by Airway Epithelial Cells

There is now considerable evidence that an important mechanism by which environmental irritants cause injury to AEC is via inducing the production of reactive oxygen species (ROS). This has been well studied in vitro in response to injury by RSV [56, 57]. Furthermore, activation of antioxidant defence mechanisms, through binding of the transcription factor Nrf2 to antioxidant response elements (AREs), has been shown to be an important part of the host response to RSV infection in vivo. RSV infection caused induction of various ARE-driven enzymes, and gene-targeted mice deficient in Nrf2 had significantly lower levels of reduced glutathione in the lungs, higher levels of oxidative modification of lung proteins and lipids, and developed significantly more severe RSV disease, both in terms of inflammation and epithelial injury [58]. Similarly, in vitro studies have demonstrated that DEP induce oxidative stress, with greater effects on AEC than on other target cells such as pulmonary macrophages [59]. There is clear evidence that paracetamol (acetaminophen) decreases intracellular levels of reduced glutathione and thus predisposes to injury by ROS [60].

Oxidative stress leads to activation of key intracellular signalling mechanisms, for example, the mitogen-activated protein kinase (MAPK) and NF-κB pathways, and these can be inhibited by administration of antioxidants [61, 62]. Activation in turn leads to the generation of chemoattractants and proinflammatory cytokines. Whether similar events occur in vivo during the induction phase of paediatric asthma has not been formally demonstrated. However, the concept that oxidant injury may be a key early event is supported by evidence that functional polymorphisms in oxidant defence genes increase the risk of developing asthma in childhood [63, 64].

Environmental irritants may also trigger AEC via pattern recognition receptors, including the predominantly cell surface or endosomal TLRs and the cytoplasmic Nod-like receptors (NLRs). These receptors are key components of host innate defences, capable of recognising conserved molecular patterns associated with pathogens or with cellular damage [65]. TLRs signal via adaptor proteins, notably MyD88, leading to activation of MAPK and NF-κB signalling pathways [66]. Whether TLR-dependent signalling promotes the development of a Th2-biased response following exposure to viral infection or environmental irritants is not altogether clear. However, the potential importance of TLR-mediated responses is supported by evidence that polymorphisms in genes for TLR2 and TLR4 are related to increased prevalence of asthma from birth up to the age of 8 years [67]. Members of the NLR family assemble into large multiprotein complexes, termed inflammasomes, which activate caspase-1, a proteolytic enzyme that cleaves and thus activates cytokines such as IL-1β and IL-18 for secretion [65]. Expression of NLRs has been demonstrated in the airway epithelium [68], but their role in the response to injury by environmental irritants, or in driving allergic inflammation, has not hitherto been investigated.

Another potentially important mechanism by which cytokine release from AEC might be triggered is via the enzymatic activity of some allergens on protease-activated receptors. For example, fungal antigens appear to be able to elicit secretion of TSLP via PAR-2 activation [69].

4. A Proasthmatic Epithelial Phenotype

An intriguing question, especially in relation to early-life viral infection, is whether environmental injury might lead to induction of a relatively stable epithelial phenotype that characterises and/or promotes the development of asthma [70]. There is no doubt that the airway epithelium of asthmatics is different to that of nonasthmatics, with evidence of abnormal proliferation/repair and enhanced production of proinflammatory cytokines [71–73]. Notably, this includes enhanced expression of TSLP and GM-CSF by asthmatic AEC [74, 75]. Furthermore, impaired production of interferons has been related to the increased susceptibility of asthmatics to viral infections [76, 77]. However, what is not clear is whether this is a reason for development of asthma, or an effect of a predisposing factor or of asthma itself. The issue is relevant to the ongoing debate about the relationship between viral infection and asthma [78]. Interestingly, recent evidence suggests that in a Th2-biased environment, which induces IL-13-driven mucous cell change in the airway epithelium, the altered epithelial lining may be more susceptible to infection by rhinovirus [79]. Thus, the mucous cell hyperplasia and other remodelling of the airway epithelium associated with the development of childhood asthma [80] may predispose to progression of the asthmatic phenotype, by promoting a vicious cycle of viral and allergic inflammation.

5. Conclusion

Injury to AEC by environmental factors may have an important role in initiating a cascade of responses that can lead to the development of asthma in childhood. Oxidant stress by environmental injurious agents may activate a variety of intracellular signalling pathways, that in turn drive the synthesis and secretion of multiple cytokines able to promote the recruitment of T cells and their polarisation towards a Th2 cytokine-secreting phenotype. However, at present there are many gaps in our understanding of the pathogenetic sequence of events. Further studies in animal models of childhood asthma may help to identify interventions that could reduce the risk of allergic asthma. Caution is warranted, however, because the mechanisms are likely to be complex and targeting single cytokines or regulatory pathways is rarely successful in asthma [81, 82].

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Research Article

IL-17F Induces CCL20 in Bronchial Epithelial Cells

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IL-17F plays a crucial role in airway inflammatory diseases including asthma, but its function has not been fully elucidated. CCL20 is also involved in allergic airway inflammation, while its regulatory mechanisms remain to be defined. To further identify a novel role of IL-17F, the expression of CCL20 by IL-17F in bronchial epithelial cells and the signaling mechanisms involved were investigated. Bronchial epithelial cells were stimulated with IL-17F, and the levels of CCL20 gene and protein measured, with the effects of the addition of various kinase inhibitors and siRNAs also investigated. IL-17F significantly induced the expression of CCL20 gene and protein. Pretreatment with inhibitors for MEK1/2, Raf1 and MSK1, and overexpression of a Raf1 dominant-negative mutant significantly diminished IL-17F-induced CCL20 production. Moreover, transfection of the siRNAs targeting MSK1, p90RSK, and CREB blocked CCL20 expression. These findings suggest that IL-17F is able to induce CCL20 via Raf1-MEK1/2-ERK1/2-MSK1/p90RSK-CREB signaling pathway in bronchial epithelial cells. The IL-17F/CCL20 axis may be a novel pharmacological target for asthma.

1. Introduction

The IL-17 family of cytokines consists of six members, IL-17 (also called IL-17A), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F [1–5]. We and other groups discovered human IL-17F [6–8]. We have reported that IL-17F is capable of inducing several cytokines and chemokines in bronchial epithelial cells [9–16]. The signaling pathway of IL-17F has been uncovered. Similar to IL-17A, the receptor for IL-17F is the heterodimeric complex of IL-17RA and IL-17RC [17]. Although human IL-17RA binds IL-17A effectively, it binds IL-17F with ~1000-fold lower affinity [18]. The relative binding affinity of IL-17F to IL-17RC is much stronger than to IL-17RA. Activation of the receptor by IL-17F leads to an interaction with Act-1 via the similar expression to fibroblast growth factor genes, IL-17 receptors, and TIR (SEFIR) domain [19]. This mediates activation of TNF receptor-associated factor (TRAF)-6 [19, 20]. Moreover, we have identified the downstream pathway of IL-17F receptor signaling. IL-17F activates the Raf1-MEK1/2-ERK1/2-MSK1/p90RSK-CREB signaling pathway [10–16]. In the airway of asthmatics, the expression of IL-17F is clearly upregulated [6], and is correlated with the disease severity [6, 21, 22]. We have also demonstrated that a coding-region variant (H161R) of the IL-17F gene is inversely associated with asthma and encodes an antagonist for the wild-type IL-17F [23, 24]. Moreover, a recent study showed that IL-17F has a possible role in the mechanism of steroid resistance in asthma [25]. These findings suggest that IL-17F is one of the important cytokines involved in the pathogenesis of allergic airway inflammation. IL-17F is derived from activated $CD4^+$ T cells, basophils, and mast cells, three key-effector cell types involved in asthma [6]. Moreover, IL-17F is produced by a recently discovered lineage of $CD4^+$ T cells, Th17 cells [26]. Th17 cells selectively produce hallmark cytokines IL-17A and IL-17F, but not IL-4 and INFγ, and they play a pivotal role in airway diseases including asthma [27]. In a mouse model of asthma, Th17 cells-mediated airway inflammation and airway hyperresponsiveness are steroid resistant [28]. In

asthmatic patients, increased numbers of tissue-infiltrating Th17 cells are observed in the airway [29]. Another study demonstrated that the number of peripheral Th17 cells is significantly elevated in asthmatic patients compared with control subjects [30]. These findings suggest that Th17 cells have a potential role in the pathogenesis of asthma. However, it is unclear how Th17 cells traffic into the airway of asthmatics.

CCL20 is a CC chemokine and a unique functional ligand for CCR6. CCL20 is derived from bronchial epithelial cells in response to several stimuli such as proinflammatory cytokines, ambient particulate matter, and the proteolytic allergen Der p1 [31–33]. CCL20 is involved in the pathogenesis of airway inflammatory diseases including asthma. Indeed, its levels are significantly elevated in bronchoalveolar lavage (BAL) fluid from patients with allergic asthma when compared with control subjects [32]. It is also reported that the CCL20/CCR6 system plays a pivotal role in allergic airway responses such as airway resistance, airway eosinophilia, and production of IL-5 and IgE [34]. In addition, a recent study demonstrated that human Th17 cells predominantly express CCR6 [35]. This implies that CCL20 is able to attract Th17 cells into the site of airway inflammation via CCR6. However, inducers of epithelium-derived CCL20 in airway inflammation and its regulatory mechanisms have not been fully understood. To this end, the effects of IL-17F on expression of CCL20 were investigated. In this study, we demonstrated, for the first time, that IL-17F induces CCL20 in bronchial epithelial cells via the activation of RafI-MEK1/2-ERK1/2-MSK1/p90RSK-CREB signaling pathway.

2. Materials and Methods

2.1. Cell Culture. Two different bronchial epithelial cells were used in this study. A bronchial epithelial cell line, BEAS-2B, was cultured in Hanks' F12/DMEM (Biofluids, Rockville, Md, USA) with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin (Life Technologies-BRL, Gaithersburg, Md, USA). Normal human bronchial epithelial cells (NHBEs) were purchased from Lonza (Walkersville, Md, USA), and cultured in bronchial epithelial basal medium according to the manufacturer's instruction. The cells were cultured for no more than 3 passages prior to the analysis.

2.2. Analysis of CCL20 Gene Expression. Total RNA was extracted using RNeasy (Qiagen, Chatsworth, Caif, USA) from 1×10^6 BEAS-2B cells at 4 hrs after stimulation with 10 and 100 ng/mL of IL-17F (R&D Systems, Minneapolis, Minn, USA). cDNAs were synthesized from 500 ng of total RNA using the cDNA synthesis kit (TOYOBO, Tokyo, Japan), followed by real-time PCR. The sequences of real-time PCR primers for CCL20: forward, 5′-CTGGCTGCTTTGATGTCAGT-3′, reverse, 5′-CGTGTGAAGCCCACAATAAA-3′; G3PDH: forward, 5′-ACCACAGTCCATGCCATCAC-3′, reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. Real-time PCR was done using a SYBR Green PCR kit (Applied Biosystems, Tokyo, Japan), gene-specific primers, and an ABI 7700 thermal cycler. The gene expression levels for each amplicon were calculated using the $\Delta\Delta C_T$ method and normalized against G3PDH gene. The data were shown as fold induction relative to the control group. The values are expressed as mean ± SEM ($n = 6$ experiments).

2.3. Analysis of CCL20 Protein Expression. Cell supernatants in BEAS-2B cells and NHBEs were harvested from cultures in the absence or presence of 10 or 100 ng/mL of IL-17F at 2, 6, 12, 24, or 48 hrs after stimulation. Alternatively, BEAS-2B cells were also stimulated with 100 ng/mL of IL-17A and IL-17E (IL-25) (R&D Systems) for 24 hrs. CCL20 protein levels in the supernatants were determined with a commercially available ELISA kit (R&D Systems) according to the manufacturer's instruction. The values are expressed as mean ± SEM ($n = 6$ experiments).

2.4. Effect of Inhibitors on the Expression of CCL20. For analysis of involvement of the Raf1-MEK-ERK1/2-MSK1 pathway, BEAS-2B cells were treated in the presence or ab-sence of the following kinase inhibitors at varying doses: MEK1/2 inhibitors, PD98059 (Calbiochem, La Jolla, Caif, USA), and U0126 (New England Bio Labs, Beverly, Mass, USA); p38MAPK inhibitor, SB202190 (Calbiochem); a Raf1 kinase inhibitor I (Calbiochem); a JNK inhibitor, SP600125 (Calbiochem); MSK1 inhibitors, H89 and Ro318220 (Calbiochem); and a vehicle control, DMSO (Me_2SO) for 1 hr before treatment with IL-17F (100 ng/mL). The supernatants were harvested at 24 hrs after stimulation for analyses with ELISA. CCL20 protein levels in the supernatants were determined as described above. The values are expressed as mean ± SEM ($n = 6$ experiments). The total number of cells and cell viability at the end of the culture period for each experiment were similar among all culture conditions, as determined by trypan blue exclusion assay, suggesting that the inhibition of IL-17F-induced CCL20 expression did not result from cytotoxicity of those inhibitors (data not shown).

2.5. Overexpression of Dominant Negative Vector for Raf1 and Ras. The plasmids encoding pCMV-RafS621A Vector (dominant negative mutant of Raf-1) and pCMV-RasN17 Vector (dominant negative mutant of Ras) cloned into pCMV and a control vector were purchased from Clontech. The plasmids were prepared by using the Qiagen plasmid DNA preparation kit. Transfection experiments utilizing primary epithelial cells were technically difficult, and an epithelial cell line, BEAS-2B, was used instead. BEAS-2B cells were cultured in 100 mm plates and were transfected by Effectene Reagent (Qiagen) according to the manufacturer's instruction. The cells were selected with 500 μg/mL of Geneticin (G418; Gibco/BRL). After selection, the cells were seeded into 6-well culture plates. The cells were near confluent, and the supernatants were then harvested at 24 hrs after stimulation with 100 ng/mL of IL-17F for analyses with ELISA. CCL20 protein levels in the supernatants were determined as described above. The values are expressed as mean ± SEM ($n = 6$ experiments).

2.6. Effect of Knockdown of p90RSK, MSK1, and CREB with siRNA. Pre-designed siRNAs for MSK1 (Bio Lad), p90RSK,

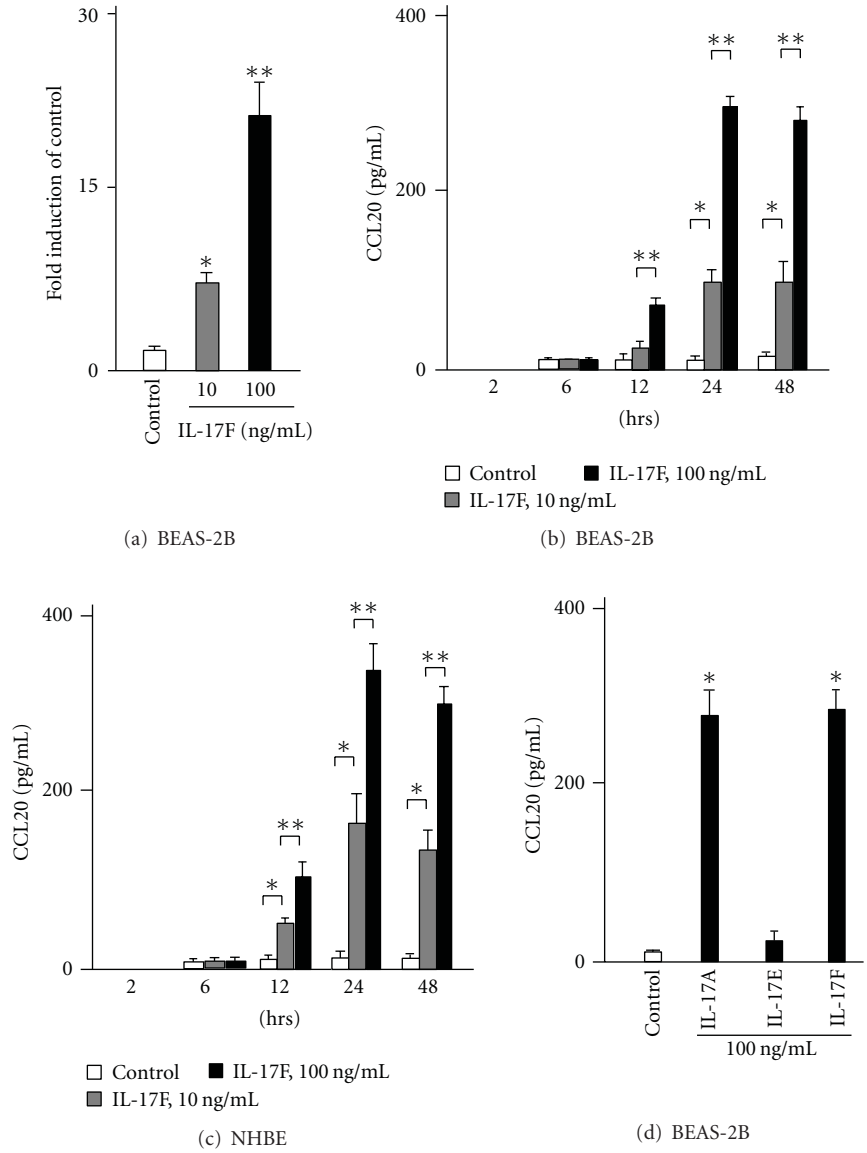

Figure 1: The expression of CCL20 gene and protein by IL-17F in bronchial epithelial cells. (a) CCL20 gene expression by real-time PCR in BEAS-2B cells. Real-time PCR was performed as described in Materials and Methods. BEAS-2B cells were stimulated with IL-17F for 4 hrs ($n = 6$). (c) CCL20 protein levels in supernatants in BEAS-2B cells. ELISA was performed ($n = 6$). (d) CCL20 protein levels in supernatants in NHBEs ($n = 6$). $^*P < .05$ versus medium control. $^{**}P < .05$ versus 10 ng/mL of IL-17F-stimulated cells. (e) CCL20 protein levels induced by IL-17A, IL-17E (IL-25), and IL-17F in supernatants in BEAS-2B cells ($n = 6$). $^*P < .05$ versus medium control. The values are expressed as means ± SEM.

CREB and, control siRNAs (Ambion, Tokyo, Japan) were used. The siRNA transfection into BEAS-2B cells was performed according to the manufacturer's instruction. The supernatants were then harvested at 24 hrs after stimulation with 100 ng/mL of IL-17F, and subjected for ELISA analyses, respectively (each $n = 6$ experiments). CCL20 protein levels in the supernatants are expressed as mean ± SEM.

2.7. Data Analysis. The statistical significance of differences was determined by analysis of variance (ANOVA). The values are expressed as mean ± SEM from independent experiments. Any difference with P values less than 0.05 was considered significant. When ANOVA indicated a significant difference, the Scheffe F-test was used to determine the difference between groups, since it is suitable for testing multiple comparisons.

3. Results

3.1. IL-17F Induces the Expression of CCL20. To examine whether IL-17F is able to induce CCL20 expression, BEAS-2B cells were stimulated with two doses of IL-17F. First, the levels of CCL20 gene expression were analyzed by real-time PCR. IL-17F significantly induced CCL20 gene expression

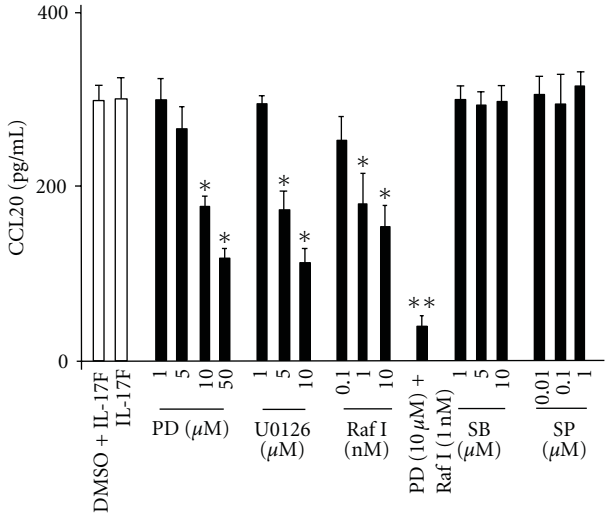

Figure 2: Effect of PD98059 (PD), U0126, Raf1 kinase inhibitor I (Raf I), SB202190 (SB), and SP600125 (SP) on CCL20 protein expression in BEAS-2Bs. The cells were pretreated for 1 hr as indicated before the 24-hr stimulation of IL-17F, and then CCL20 protein levels in supernatants were measured by ELISA. The values are expressed as means ± SEM ($n = 6$). $^*P < .05$ versus IL-17F-stimulated cells in the absence of the inhibitor. $^{**}P < .05$ versus the presence of individual inhibitor.

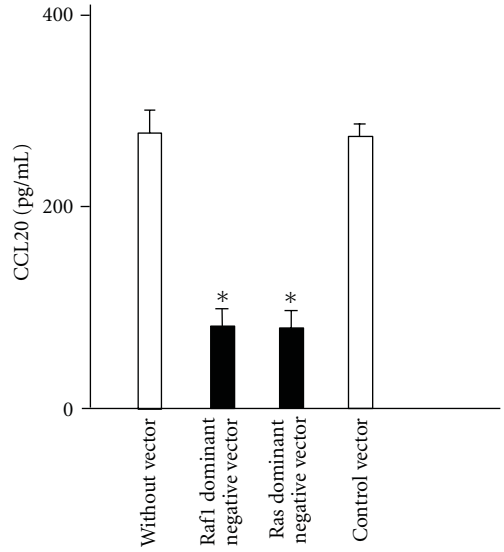

Figure 3: Effect of overexpression of dominant-negative mutants Raf1 and Ras on CCL20 protein expression in BEAS-2B cells. The cells overexpressing dominant-negative mutants were stimulated with IL-17F for 24 hrs, and CCL20 protein levels in supernatants were measured by ELISA. The values are expressed as means ± SEM ($n = 6$). $^*P < .05$ versus IL-17F-stimulated cells without vector.

in a dose dependent manner when compared with control (Figure 1(a)). Next, to analyze the protein expression for CCL20, the cells were cultured in the presence or absence of two different doses of IL-17F at five different time points. CCL20 proteins were not detected at the 2 hr time point and were weakly detected at the 6 hr time point. However, its protein levels in supernatants were significantly increased and peaked at the 24 hr time point (Figure 1(b)). Similarly, NHBEs also induced CCL20 expression in response to IL-17F and showed kinetics similar to those of BEAS-2B cells (Figure 1(c)). Another IL-17 family cytokine, IL-17A, showed similar potency in induction of CCL20 expression compared to IL-17F (Figure 1(d)). In contrast, IL-17E (IL-25) did not increase its expression in BEAS-2B cells.

3.2. MEK Inhibitors and Raf1 Kinase Inhibitor Inhibit IL-17F-Induced CCL20 Expression. Pretreatment of the cells for 1 hr with each of the selective MEK inhibitors, PD98059 (10 and 50 μM) and U0126 (5 and 10 μM), and Raf1 kinase inhibitor I (1 and 10 nM) significantly decreased the levels of IL-17F-induced CCL20 expression in BEAS-2B cells, while 1 hr pretreatment of the cells with vehicle alone (0.1% DMSO) did not affect CCL20 expression. In addition, the protein levels of CCL20 were unchanged in IL-17F-treated cells in the presence of varying doses of a p38 MAPK inhibitor, SB202190, and a JNK inhibitor, SP600125 (Figure 2). While induction of CCL20 is partially inhibited by PD98059, U0126, or Raf1 kinase inhibitor I even at relatively high dose (50 μM, 10 μM and 10 nM, resp.), the combination of 10 μM of PD98059 and 1 nM of Raf1 kinase inhibitor I inhibited, to a significant degree, the production of CCL20 (Figure 2).

3.3. Raf1 and Ras Dominant Negative Mutants Block IL-17F-Induced CCL20 Expression. Overexpression of Raf1 and Ras dominant negative mutants in BEAS-2B cells significantly inhibited IL-17F-induced CCL20 expression (Figure 3), whereas the cells transfected with a control vector showed no significant effect in the level of CCL20 production.

3.4. MSK1 Inhibitors Inhibit IL-17F-Induced CCL20 Expression. Next, to determine whether MSK1 affects IL-17F-induced CCL20 expression, the effects of MSK1 inhibitors were investigated. Pretreatment with two different MSK1 inhibitors, Ro-31-8220 and H89, significantly suppressed IL-17F-induced CCL20 expression (Figure 4).

3.5. siRNAs Targeting p90RSK, MSK1, and CREB Inhibit IL-17F-Induced CCL20 Expression. Finally, the effect of siRNA targeting p90RSK, MSK1, and CREB on the induction of CCL20 expression by IL-17F was analyzed. As shown in Figure 5, its expression by IL-17F was significantly inhibited in cells transfected with siRNA targeting p90RSK, MSK1, and CREB, while no significant difference was seen in wild-type BEAS-2B cells and cells transfected with a control siRNA.

4. Discussion

In this paper, we demonstrated, for the first time, that IL-17F induces the expression of CCL20 in bronchial epithelial cells through the activation of the Raf1-MEK-ERK1/2-p90RSK/MSK1-CREB signaling pathway. These findings suggest that IL-17F is a potent inducer of CCL20, and the IL-17F/CCR20 axis may provide new insights into the pathophysiology of asthma.

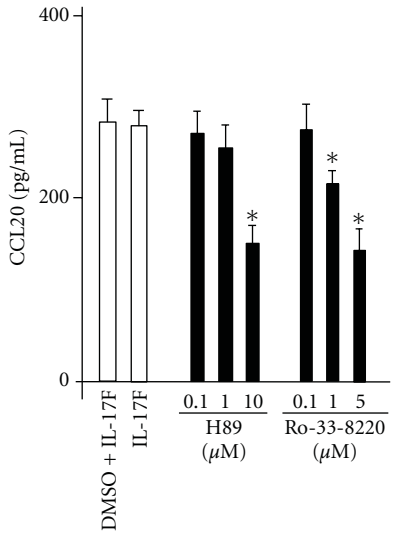

Figure 4: Effect of MSK1 inhibitors, H89, and Ro318220 on IL-17F-induced CCL20. BEAS-2B cells were pretreated for 1 hr as indicated before the 24-hr stimulation of IL-17F, and then CCL20 protein levels in supernatants were measured by ELISA. The values are expressed as means ± SEM ($n = 6$). $^*P < .05$ versus IL-17F-stimulated cells in the absence of the inhibitor.

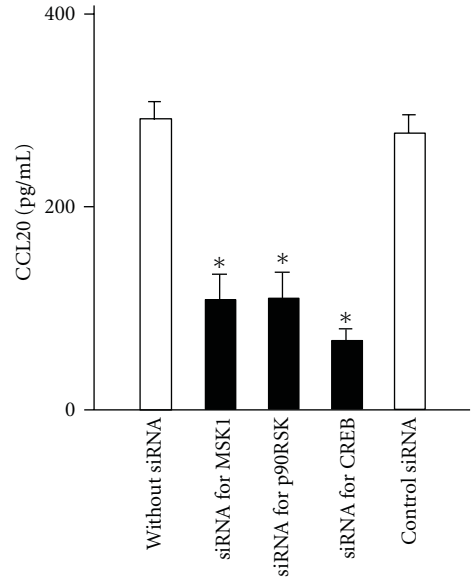

Figure 5: Effect of siRNA for p90RSK, MSK1 and CREB on IL-17F-induced CCL20 expression. BEAS-2B cells transfected with siRNAs as indicated were stimulated with IL-17F for 24 hrs, and then CCL20 protein levels in supernatants were measured by ELISA. The values are expressed as means ± SEM ($n = 6$). $^*P < .05$ versus non-transfected cells.

IL-17F is potentially involved in the pathogenesis of asthma. Expression of the IL-17F gene is upregulated in BAL cells from asthmatics following segmental allergen challenge [6]. Its expression was seen in both bronchial epithelium and inflammatory infiltrates in asthmatic patients [21, 22]. Immunocytochemistry showed that IL-17F positive cells in the subepithelial component and epithelium are significantly elevated in severe asthma compared with control and mild asthmatic subjects [22]. Furthermore, a polymorphism in *IL-17F* gene that results in a loss of lung function mutation is inversely related to asthma risk [23, 24]. In the mouse model of asthma, IL-17F is clearly expressed in the lung [36] and is able to cause pulmonary neutrophilia and provides an additive effect on antigen-induced allergic inflammatory responses [37]. IL-17F exerts multiple functions. *In vitro*, we have demonstrated that IL-17F stimulates bronchial epithelial cells to induce numerous cytokines and chemokines such as IL-6, IL-8, IL-11, ENA-78, GROα, GM-CSF, IP-10, and IGF-I [6, 9–16]. Furthermore, IL-17F is capable of inducing several cytokines and chemokines in eosinophils and lung structural cells including vein endothelial cells and fibroblasts [8, 38]. These cell types may play crucial roles in asthma in response to IL-17F. Prior to this study, it was unknown whether IL-17F affects CCL20 expression. In this paper, we have found, for the first time, that IL-17F is a potent inducer of CCL20 in bronchial epithelial cells. IL-17F shows the highest homology with IL-17A among the IL-17 cytokine family [6]. In this study, IL-17A is also able to induce CCL20 in bronchial epithelial cells, and this is consistent with the previous study [39]. Similarly to IL-17F, IL-17A produced CCL20 via the phosphorylation of ERK1/2, but not p38MAPK and JNK. Moreover, IL-17A activated NF-κB as the downstream of ERK1/2. In contrast, we reported that IL-17F is not able to activate NF-κB in bronchial epithelial cells [12]. At present, little is known about the difference of signaling pathway for IL-17A and IL-17F. Additional work is needed to determine their regulatory mechanisms.

CCL20 has a pivotal role in the pathogenesis of asthma, and is strongly derived from bronchial epithelial cells in response to a broad spectrum of asthma-related stimuli such as pro-inflammatory cytokines, ambient particulate matter, and the proteolytic allergen Der p1 [31–33]. In asthmatic patients, the level of CCL20 is significantly elevated in BAL fluid when compared with control subjects, and is more increased after endobronchial allergen provocation [32, 40]. Moreover, significant increase in its expression in BAL cells from subjects with corticosteroid-resistant asthma was seen when compared with those with corticosteroid-sensitive asthma [41]. In the mouse model of asthma, the CCL20/CCR6 system plays a pivotal role in allergic airway responses such as airway resistance, airway eosinophilia, and production of IL-5 and IgE [34]. Interestingly, CCR6 is predominantly expressed on human Th17 cells, and CCL20 shows chemotactic activity for Th17 cells [35, 42]. Emerging evidence suggests that Th17 cells are implicated in the pathogenesis of asthma [27]. Although little is known about how Th17 cells migrate into the airway, the current study suggests that IL-17F is able to attract Th17 cells into the site of airway inflammation via, at least partially, the CCL20/CCR6 system. Taken together, it is possible that IL-17F-induced epithelial CCL20 attracts Th17 cells into the airway, and accumulated Th17 cells establish a positive feedback loop

resulting in the recruitment of additional Th17 cells via the inducing IL-17F. On the other hand, Th17 cells may not be the major cell source of IL-17F in airway diseases [43]. Indeed, IL-17F is produced by various cell types, such as basophils, mast cells, monocytes, memory $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta$T cells, and NKT cells [6, 8, 44, 45]. Hence, these IL-17F-producing cells may exert an effect on bronchial epithelial cells to induce CCL20 and attract Th17 cells via CCL20/CCR6 system. The IL-17F/CCL20 axis might be especially important in the pathophysiologic events of allergic airway inflammation. However, further *in vivo* study is needed to clarify the importance of the IL-17F/CCL20 axis in asthma.

The signaling pathway of IL-17F has become clearer. We have previously demonstrated that the expression of IL-17F-induced cytokines and chemokines is dependent on the activation of ERK1/2, but not p38MAPK and JNK, in bronchial epithelial cells [9–13]. The signaling mechanisms of CCL20 expression are not yet fully understood. Here, we have identified, for the first time, that IL-17F-induced CCL20 expression is through the Raf1-MEK1/2-ERK1/2 pathway, since Raf1 kinase inhibitor and MEK1/2 inhibitors significantly decreased its expression. On the other hand, it is reported that CCL20 expression in bronchial epithelial cells is mediated by ERK1/2 and p38MAPK [31]. However, in this study, IL-17F-induced CCL20 expression is mediated by ERK1/2, but not p38MAPK, since p38MAPK inhibitor SB202190 did not affect its expression. This difference may be due to the stimuli used. Therefore, ERK1/2 may be a crucial signaling molecule for the IL-17F-induced CCL20 expression. These findings suggest that regulation of the Raf1-MEK1/2-ERK1/2 pathway may constitute a useful therapeutic target for IL-17F-associated diseases including asthma. In addition, we have recently identified that MSK1-CREB and p90RSK-CREB are two critical signaling pathways of IL-17F as the downstream elements of the Raf1-MEK1/2-ERK1/2 kinase cascade [14, 15]. These pathways are essential for CCL20 expression by IL-17F, since MSK1 inhibitors, Ro-31-8220 and H89, and siRNA targeting MSK1, p90RSK, and CREB significantly diminished its expression. Hence, CCL20 expression is mediated by the RafI-MEK1/2-ERK1/2-MSK1/p90RSK-CREB signaling pathway in the case of IL-17F in bronchial epithelial cells. These data suggest that this signaling pathway is a potential pharmacological target in the IL-17F-mediated airway inflammation. However, all inhibitors used in this study did not completely abrogate IL-17F-induced CCL20 expression. This suggests that the potential involvement of other signaling pathway. Further study is needed to clarify a novel signaling molecule of IL-17F.

In conclusion, we identified a novel function of IL-17F. IL-17F is capable of inducing CCL20 in bronchial epithelial cells, and its expression is mediated by the Raf1-MEK1/2-ERK1/2-MSK1/p90RSK-CREB signaling pathway. Taken together, the IL-17F/CCL20 axis may have an orchestrating role in the pathogenesis of asthma and could possibly provide a valuable therapeutic target for development of new strategies to treat asthma.

Abbreviations

CREB: Cyclic AMP response element binding protein
ERK1/2: Extracellular signal-regulated kinase
MAPK: Mitogen-activated protein kinase
MEK: MAP kinase
MSK1: Mitogen- and stress-activated protein kinase1
NHBE: Normal human bronchial epithelial cell
p90RSK: p90 ribosomal S6 kinase.

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Review Article

Immunopathology and Immunogenetics of Allergic Bronchopulmonary Aspergillosis

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Allergic bronchopulmonary aspergillosis (ABPA) is a Th2 hypersensitivity lung disease in response to *Aspergillus fumigatus* that affects asthmatic and cystic fibrosis (CF) patients. Sensitization to *A. fumigatus* is common in both atopic asthmatic and CF patients, yet only 1%–2% of asthmatic and 7%–9% of CF patients develop ABPA. ABPA is characterized by wheezing and pulmonary infiltrates which may lead to pulmonary fibrosis and/or bronchiectasis. The inflammatory response is characterized by Th2 responses to *Aspergillus* allergens, increased serum IgE, and eosinophilia. A number of genetic risks have recently been identified in the development of ABPA. These include HLA-DR and HLA-DQ, IL-4 receptor alpha chain (*IL-4RA*) polymorphisms, *IL-10 −1082GA* promoter polymorphisms, surfactant protein A2 (*SP-A2*) polymorphisms, and cystic fibrosis transmembrane conductance regulator gene (*CFTR*) mutations. The studies indicate that ABPA patients are genetically at risk to develop skewed and heightened Th2 responses to *A. fumigatus* antigens. These genetic risk studies and their consequences of elevated biologic markers may aid in identifying asthmatic and CF patients who are at risk to the development of ABPA. Furthermore, these studies suggest that immune modulation with medications such as anti-IgE, anti-IL-4, and/or IL-13 monoclonal antibodies may be helpful in the treatment of ABPA.

1. Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disease due to bronchial colonization by *Aspergillus fumigatus* that occurs in susceptible patients with asthma and cystic fibrosis (CF). The first published description of ABPA as an entity came from the United Kingdom in 1952 [1], while the first cases in the United States were reported a decade later [2, 3]. ABPA affects approximately 1%–2% of asthmatic patients and 7%–9% of CF patients [4–6]. If unrecognized or poorly treated, ABPA leads to airway destruction, bronchiectasis, and/or pulmonary fibrosis, resulting in significant morbidity and mortality.

The diagnosis of ABPA is based on clinical and immunologic reactivity to *A. fumigatus*. The minimal criteria required for the diagnosis of ABPA are: (1) asthma or cystic fibrosis with deterioration of lung function, for example, wheezing, (2) immediate *Aspergillus* skin test reactivity, (3) total serum IgE $\geq$ 1000 IU/mL, (4) elevated *Aspergillus* specific IgE and IgG antibodies, and (5) chest radiographic infiltrates. Additional criteria may include peripheral blood eosinophilia, *Aspergillus* serum precipitating antibodies, central bronchiectasis, and *Aspergillus* containing mucus plug production [7–11]. The designation of ABPA-seropositive (ABPA-S) may be used to classify asthmatic patients who meet the required criteria but lack the proximal or central bronchiectasis (ABPA-CB). High-resolution computed tomography (HRCT) may demonstrate central bronchiectasis in the inner two thirds of the field even in the absence of chest radiograph lesions. The clinician should note that the development of ABPA is not dependent on asthma severity. The diagnosis of ABPA in CF is more complicated and disagreement exists in the literature regarding the diagnostic criteria. The difficulty lies in the fact that the usual criteria for ABPA and the common signs and symptoms of CF overlap. The most recent Cystic Fibrosis Foundation Consensus Conference proposed the following diagnostic criteria: (1) acute or subacute pulmonary deterioration not attributable to another etiology,

(2) total serum IgE >1000 IU/mL, (3) immediate cutaneous reactivity to *Aspergillus* or *in vitro* specific IgE antibodies to *Aspergillus*, and (4) one of the following: *Aspergillus* serum precipitins, elevated specific IgG anti-*Aspergillus* antibodies, new or recent chest radiographic, or chest CT abnormalities that have not cleared with antibiotics and chest physiotherapy [12].

1.1. Radiographic and Laboratory Investigations. There are several characteristic radiographic abnormalities associated with ABPA [7–11]. The most common lesion is a large, homogeneous shadow in one of the upper lobes with no change in volume. The shadow may be triangular, lobar, or patchy, and it frequently moves to another site. "Tram-line" shadows are fine parallel lines radiating from the hila that represent inflammation of airway walls. Mucoid impaction causes toothpaste shadows or gloved-finger shadows, which can be seen on plain radiograph.

Adult patients have been reported with normal chest radiographs so radiographic abnormalities are not invariably present. In these individuals, HRCT scan may reveal central cylindrical bronchiectasis even in the absence of chest radiograph abnormalities. Sometimes, "tree-in-bud pattern" may been seen on HRCT scan that indicates some degree of airway mucus plugging. It is more commonly considered evidence of atypical mycobacterial infection and may be seen in cystic fibrosis. However, central bronchiectasis is a common complication and finding in all CF patients.

Laboratory tests that support the diagnosis of ABPA are those that demonstrate allergy to the *A. fumigatus*, such as elevated specific IgE anti-*Aspergillus* antibodies and positive *Aspergillus* precipitins [7–11]. Culture of *A. fumigatus* from the sputum is only a secondary criterion for the diagnosis of ABPA, because a large proportion of individuals with CF without ABPA have *Aspergillus* on sputum cultures. Some normal individuals and many individuals with lung diseases have small numbers of spores in their sputum; these are probably present because of passive inhalation. The presence of hyphae is more specific, and the presence of eosinophils in association with hyphal elements is suggestive of the diagnosis. At the time of radiographic exacerbation, the presence of eosinophilia in sputum or blood is suggestive of ABPA in asthmatics and is a primary diagnostic criterion. The peripheral blood eosinophil count is usually greater than 1000/mm^3, and values greater than 3000/mm^3 are common. Eosinophilia is not a diagnostic criteria of ABPA in CF patients. In the authors' experience, eosinophilia is an uncommon finding in CF ABPA patients.

An increased total serum IgE level is very characteristic of ABPA, and values may reach as high as 30,000 IU/mL. Usually, the level is greater than 1000 IU/mL. Much of the IgE is not specific to *Aspergillus* but is the result of polyclonal B-cell activation. The IgE level is a very useful marker of disease activity, and it can be used to follow outpatients for "flares". The simple skin-prick test is a useful screening test, as ABPA is very unlikely in patients with a negative reaction. A dual-reaction skin test with an immediate (10–15 minutes) and a late (4–8 hours) reaction may occur but is uncommon in ABPA. Alternatively, serum may be measured for the presence of specific IgE and IgG antibodies. Patients with *Aspergillus*-sensitive asthma will generally have elevated *Aspergillus*-specific IgE antibodies, but patients with ABPA will have much higher *Aspergillus*-specific IgE levels. Hemmann et al. [13] reported that ABPA and *Aspergillus*-sensitive patients have elevated IgE antibodies to recombinant *Aspergillus* Asp f1, Asp f3, Asp f4, and Asp f6 allergens and that IgE levels to Asp f4 and Asp f6 is highly specific for ABPA in CF patients.

Differentiating between a bacterial flare versus an ABPA flare in CF patients may be difficult. A useful serum biologic marker may be thymus and activation-regulated chemokine (TARC) or CCL17. Latzin et al. [14] and Hartl et al. [15] reported that TARC was elevated in CF patients with ABPA and was further elevated during acute flares of ABPA. TARC is a chemokine whose ligand is CCR4 receptor on CD4$^+$ Th2 cells.

In ABPA, immunoelectrophoresis generally shows one to three precipitin lines, often to only one extract [7–11]. Patients with aspergilloma will have multiple precipitin lines to all antigen extracts. Extracts of *A. fumigatus* contain a complex mixture of proteins that are mainly derived from the hyphae. Antigenic composition varies between batches according to the culture conditions even within the same laboratory. There is, therefore, a lack of standardization that makes it difficult to compare results between laboratories. There are 22 recognized recombinant allergens by the International Union of Immunological Societies. With purification of these major antigenic components, this may lead to improved diagnosis.

1.2. Pulmonary Pathology of ABPA. The gross pathology of ABPA demonstrates cylindrical bronchiectasis of the central airways, particularly those to the upper lobes [7–11]. These airways may be occluded by "mucoid impaction," a condition in which large airways are occluded by impacted mucus and hyphae. Airway occlusion may lead to atelectasis of a segment or lobe and, if the atelectasis is long-standing, saccular bronchiectasis may result. Typically, ABPA is worse in the upper lobes than in the lower lobes. Microscopic examination of the airways shows infiltration of the airway wall with eosinophils, lymphocytes, and plasma cells. The airway lumen may be occluded by mucus containing hyphal elements and inflammatory cells, especially eosinophils. Squamous metaplasia of the bronchial mucosa commonly develops, and granulomas may form. Rarely, bronchiolitis obliterans or bronchocentric granulomatosis develops.

2. Immunopathogenesis of ABPA

As seen in Figure 1, the pathogenesis of ABPA in susceptible persons begins with the inhalation of *A. fumigatus* spores that germinate into hyphae deep within the bronchi. Fragments of hyphae have also been found within the lung parenchyma, potentially resulting in high concentrations of *Aspergillus* allergens exposed to the respiratory epithelium and immune system [16–19]. These allergens are processed by HLA-DR2 or HLA-DR5 bearing antigen presenting cells (APCs) and presented to T cells within bronchoalveolar lymphoid tissue

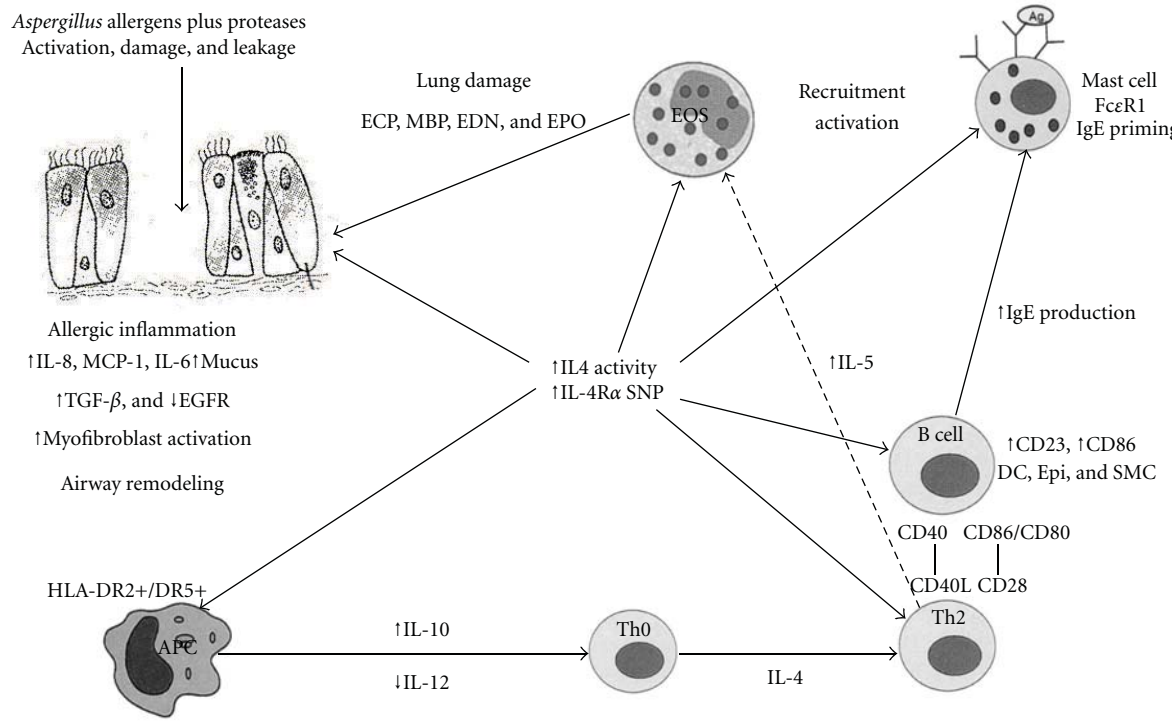

Figure 1: Proposed immunopathogenesis of ABPA. In the pathogenesis of ABPA, *A. fumigatus* proteases have a direct effect on bronchial epithelia causing epithelial cell damage with subsequent stimulation of cytokines and chemokines. *Aspergillus* proteins are processed via HLA-DR2/DR5 bearing dendritic cells that skew the Th0 response to a Th2 response. Th2 cytokines stimulate IgE synthesis and eosinophil activation. This leads to an eosinophilic inflammatory in the bronchial airways.

(BALT). The resulting $CD4^+$ T cell responses to *Aspergillus* are skewed toward Th2 response with the production of IL-4, IL-5, and IL-13 cytokines.

2.1. Effect of Aspergillus on Bronchial Epithelium. *A. fumigatus* spores 3 to 5 μm in size are inhaled and germinate deep within the bronchi into hyphae [17]. In addition, fragments of the hyphae can be identified within the interstitial of the pulmonary parenchyma. The implication of this is that there is the potential for high concentrations of *A. fumigatus* allergens exposed to the respiratory epithelium and immune system. *A. fumigatus* releases a variety of proteins, including superoxide dismutases, catalases, proteases, ribotoxin, phospholipases, hemolysin, gliotoxin, phthioic acid, and other toxins. The first line of defense against *Aspergillus* colonization in the lungs is macrophage and neutrophil killing of the conidia and the hyphae. In the development of ABPA, Kauffman's group proposed that *Aspergillus* proteins have a direct effect on the pulmonary epithelia and macrophage inflammation [20, 21]. They demonstrated that *Aspergillus* proteases induce epithelial cell detachment. In addition, protease-containing culture filtrates of *Aspergillus* induce human bronchial cell lines to produce proinflammatory chemokines and cytokines, such as IL-8, IL-6, and MCP-1. Thus, various *Aspergillus* proteins have significant biologic activity that disrupts the epithelial integrity and induces a monokine inflammatory response. This protease activity is thought to allow for enhanced allergen exposure to the bronchoalveolar lymphoid tissue immune system. This is evident by the bronchoalveolar lymphoid tissue synthesis of *Aspergillus*-specific IgE and IgA antibodies.

An important pathogenic feature of *Aspergillus* and other microbes is their ability to interact with epithelial cells on the mucosal surface. Macrophage and neutrophil killing of the conidia and hyphae is the first line of defense against colonization in the lungs [12, 22–24]. This is evidenced by an increased susceptibility to invasive pulmonary aspergillosis in patients with chronic granulomatous disease, a disorder of phagocyte killing. *A. fumigatus* has several virulence factors, including proteolytic enzymes that can interfere with humoral and cellular defense in the airways [25, 26]. Proteases from *Aspergillus* and other fungi, including *Alternaria* and *Cladosporium*, have been shown to cause epithelial cell detachment though *Aspergillus* proteases demonstrated more activity at lower concentrations [25–28].

In addition to damaging the integrity of the epithelial cell layer, Kauffman's group demonstrated that protease containing culture infiltrates of *A. fumigatus* induced human bronchial cell lines to produce proinflammatory chemokines and cytokines, such as monocyte chemoattractant protein (MCP)-1, IL-8, and IL-6 [20]. MCP-1 has been implicated in directly stimulating the development of Th2 cells [29]. The cytokine-release activity could be ascribed to the proteolytic activities of these extracts [20, 27]. These observations suggested that proteolytic enzymes released by *Aspergillus*, growing on and between epithelial cells, were responsible for

the induction of chemoattractive cytokines by epithelial cells and the corresponding inflammation. It was proposed that the induction of the severe inflammatory responses by the direct activation of epithelial cells may cause additional harm to the epithelial cell layer [25]. Destruction of the epithelial cell barrier either by fungal proteases or eosinophilic and neutrophilic inflammation was followed by repair mechanisms, resulting in the influx of serum proteins and extracellular matrix proteins to the luminal side of the epithelium [30]. Because spores and mycelium of *A. fumigatus* have surface structures that are able to interact with extracellular matrix molecules, damage and repair mechanisms of the airway mucosa may facilitate the binding of *Aspergillus* to the damaged sites of the airways. The enhanced release of proteolytic enzymes and allergens on the epithelial surface would induce a continuous inflammatory response and mast cell degranulation, resulting in severe and long-lasting periods of exacerbations of ABPA.

2.2. Aspergillus-Specific Th2 Cells. The immune response to *Aspergillus* antigens in ABPA patients, as well as allergic asthmatic and CF patients, is characterized by a Th2 $CD4^+$ T lymphocyte response [17, 31–35]. Skin test reactivity to *Aspergillus* is found in 20%–25% of asthmatic patients [5, 36, 37] and 31%–59% of CF patients [13, 38]. Although sensitization is common in these populations, only a small percentage of patients develop ABPA.

Several groups have observed T cell lymphoproliferative responses to crude *Aspergillus* extracts [31, 39–41]. Subsequently, *Aspergillus*-specific T cell responses were examined and shown to enhance B cell IgE synthesis [41]. In addition, Asp f1 T cell lines were generated, and the phenotypes were found to be $CD4^+$ $CD25^+$ T cells with the cytokine profile IL-4^+ and IFNγ^-, indicating Th2 $CD4^+$ T cells [4]. Chauhan et al. [42] subsequently developed T cell clones from asthmatic ABPA patients and demonstrated either Th2 (IL-4^+, IFN-γ^-) or Th0 (IL-4^+, IFN-γ^+) patterns. We demonstrated that ABPA subjects have increased frequency of IL-4^+ $CD3^+$ T cells from Asp f2/f3/f4-stimulated peripheral blood lymphocytes compared to *Aspergillus* sensitive non-ABPA subjects [4]. IL-4 produced by T lymphocytes binds to the IL-4 receptor (IL-4R) on B cells and in association with the CD40L/CD40 signals, results in IgE isotype switching and B cell proliferation [43]. IL-4 also increases the expression of CD86, which has been linked to eosinophilic airway inflammation and airway hyperresponsiveness after allergen challenge. A central question then is how ABPA patients differ from *Aspergillus*-sensitive atopic asthmatic and CF patients. We hypothesize that ABPA develops in genetically susceptible individuals with asthma and CF because of increased frequency and/or activity of *A. fumigatus*-specific Th2 $CD4^+$ cells. We further propose that polymorphisms of the interleukin-4 receptor alpha chain (*IL-4RA*) subunit and HLA-DR2/DR5 are the genetic susceptibility risk factors responsible for the development of ABPA.

2.3. IL-4 Responses in ABPA. Human studies and murine models have shown that $CD4^+$ Th2 cells and their cytokines are central to the development of ABPA [4, 32–35, 39, 44]. In particular, IL-4 has a key role in the allergic inflammatory response with effects on various cell populations. Its functions include increasing VCAM-1 expression on endothelial cells, which enhances the recruitment of other immune cells, particularly eosinophils, stimulating proliferation of fibroblasts, important in airway remodeling, and increasing Th2 differentiation while decreasing Th1 differentiation and the production of IFN-γ[45, 46]. IL-4 also has a myriad of effects on B lymphocytes including the stimulation of growth and activation, increasing HLA-DR class II expression important for antigen presentation and inducing cell surface expression of CD23 and soluble CD23. This cell surface molecule is the low affinity IgE receptor (FcεRII) and an activation marker present on a number of cells including B cells, activated T cells, monocytes, and eosinophils. CD23 plays a role in augmenting B cell IgE synthesis through its interactions with CD21 [47, 48]. Recently, in 2003, anti-CD23 monoclonal antibody was administered to atopic asthmatic subjects and resulted in decreased serum IgE levels [49]. In addition, IL-4 has a more direct role in IgE isotype switching by B-cells. It should be noted that IL-13 may also stimulate the synthesis of IgE and is the only other cytokine that has this capability [50–52]. Recently, in 2000 and 2004, increased sensitivity to *in vitro* IL-4 stimulation as measured by enhanced expression of the low-affinity IgE receptor (CD23) on B cells was observed in ABPA patients [32, 33]. This was associated with single-nucleotide polymorphisms of the IL-4 receptor alpha chain (*IL-4RA*) in 92% of ABPA subjects, principally the IL-4-binding single-nucleotide polymorphism ile75val [19, 34, 35]. This increased sensitivity to IL-4 is demonstrated by increased expression of CD23 and CD86 on B cells of ABPA subjects and increased CD23 expression during flares of ABPA [19]. CD23 is expressed on a variety of cells, including B cells, natural killer cells, subpopulations of T cells, and a subpopulation of dendritic cells. T-cell CD23 and B-cell CD21 form a costimulatory pathway. T-cell CD28, B-cells CD80, and CD86 costimulatory pathways activate both T and B cells, and CD28:CD86 is important in IgE synthesis. CD86 is also found on dendritic cells that have the histamine receptor 2, which skews antigen-specific T cells to a Th2 response. We have also observed increased CD86 expression on monocyte-derived dendritic cells of ABPA subjects. Thus, antigen-presenting cells such as monocytes and dendritic cells bearing HLA-DR2 and/or HLA-DR5 and increased sensitivity to IL-4 stimulation probably play a critical role in skewing *A. fumigatus*-specific Th2 responses in ABPA.

3. Immunogenetics of ABPA

3.1. HLA-DR and HLA-DQ. HLA-DR restriction has been shown to be a risk factor for the development of ABPA (Table 1). Chauhan et al. [42, 53] observed that asthmatic and CF patients who expressed HLA-DR2 and/or DR5 but lacked HLA-DQ2 were at increased risk for ABPA after exposure to *A. fumigatus*. Within HLA-DR2 and HLA-DR5, there are restricted genotypes. In particular, HLA-DR2 HLA-DRB1*1501 and HLA-DRB1*1503 genotypes were reported to provide high relative risk. On the other hand, 40% to 44% of non-ABPA atopic *Aspergillus*-sensitive individuals have

Table 1: Genetic risk factors in the development of allergic bronchopulmonary aspergillosis.

(i) HLA-DR restriction and HLA-DQ protection
(a) HLA-DR2 restriction
HLA-DRB1*1501 and *HLA-DRB1*1503
(b) HLA-DR5
HLA-DRB1*1104
(c) HLA-DQ2 protective, decreased in ABPA
DQB1*0201
(ii) *IL-4RA* polymorphisms
(a) *IL-4RA* ile75val
(iii) IL-10 polymorphisms
(a) Promoter −1082 GG genotype
(iv) Surfactant protein A2 (*SP-A2*) polymorphisms
(a) *SP-A2* ala91pro
(v) Cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations
(a) Heterozygous *CFTR* mutations in asthmatic patients with ABPA
(vi) Toll-like receptor (*TLR*) polymorphisms
(a) *TLR9* T-1237C polymorphism

the HLA-DR2 and/or DR5 type. Further studies indicated that the presence of HLA-DQ2, especially DQB1*0201, provided protection from the development of ABPA. Furthermore, Chauhan et al. [42] demonstrated that Asp f1 allergen has a low-affinity of binding to HLA-DR. This is consistent with Th2 T cell response previously reported by others in that strong antigen HLA-DR-Ag-TCR affinity binding favored a Th1 cellular response, whereas low affinity binding favored a Th2 humoral response [54–58]. Four major Vβ chains, Vβ 3, 6, 13, and 14, reacted to Asp f1.

3.2. IL-4 Alpha Chain Receptor (IL-4RA) Polymorphisms. The IL-4 receptor is a type I cytokine receptor and exists as a heterodimer that shares a subunit, IL-4 receptor alpha chain (IL-4RA), with the IL-13 receptor alpha (IL-13RA) [59]. There are two types of IL-4 receptors. Type I receptors, found on all lymphohematopoietic cells, are composed of the IL-4RA and the common gamma chain (γC), which is also a component of IL-2, IL-7, IL-9, IL-15, and IL-21 cytokine receptors [60]. IL-4 receptor type II, also known as the IL-13 receptor, is formed by the association of IL-4RA with the IL-13RA subunits and is located on immune cells, bronchial epithelium, and vascular endothelium. IL-4 stimulates both type I and type II receptors, while IL-13 signals through type II receptors.

A potential gain-of-function in the IL-4RA subunit may be responsible for B cell hyperreactivity in ABPA. As a consequence of increased IL-4R activity, proinflammatory cytokines skew T cell responses to a dominant Th2 pattern which ultimately contributes to the pathophysiology and progression of ABPA. There are eight naturally occurring single nucleotide polymorphisms (SNPs) of the *IL4RA* gene: ile75val, glu400ala, cys431arg, ser436leu, ser503pro, gln576arg, ser752ala, and ser786pro reported thus far [61–71]. Chromosome 16, which has been associated with asthma, contains the *IL-4RA* gene [66]. Studies have identified a number of these SNPs to be associated with atopy prevalence and asthma severity. In 1997, Hershey et al. [62] initially reported on a high prevalence of atopy and a gain-of-function in the IL-4RA as measured by increased CD23 expression in patients with gln576arg and a later study found that this allele correlated with asthma severity [68]. Hershey's group found that the presence of two variants, val75 and arg576 together, resulted in elevated IL-4 dependent CD23 expression which was not observed when these SNPs were present alone [71]. In our studies, the presence of the val75 allele, located within the IL-4 binding region, was found in 87.5% of ABPA subjects examined, while the cytoplasmic SNPs were present much less frequently at 27.3% for ala400, 27.3% pro503, 27.3% arg576, and 9.1% arg431. Although these alleles, particularly val75, appear to be common in the general population, their high prevalence in ABPA suggests that they may be a risk factor in the development of the disease (Table 1).

3.3. IL-10 Polymorphisms. Brouard and coworkers [72] recently in 2005 reported another genetic risk, the association of the *−1082GG* genotype of the IL-10 promoter with colonization with *A. fumigatus* and the development of ABPA in CF (Table 1). The *−1082GG* polymorphism has been associated with increased IL-10 synthesis, whereas the *−1082A* allele has lower IL-10 synthesis. Thus, dendritic cells expressing HLA-DR2/DR5, increased IL-10 synthesis and increased sensitivity to IL-4 stimulation due to *IL-4RA* polymorphisms, may be responsible for skewing *Aspergillus*-specific Th2 responses in ABPA.

3.4. Surfactant Protein A2 (SP-A2) Polymorphisms. Recently, in 2003, Saxena et al. [73] reported that ABPA patients with polymorphisms (ala91pro and arg94arg) in the collagen region of pulmonary surfactant protein A2 (*SP-A2*) had more elevated total IgE levels and higher percentages of eosinophilia than observed in those patients who lacked the SNPs (Table 1). They also found that 80% of patients carrying both alleles had ABPA ($P = 0.0079$, OR = 10.4), while only 50% and 60% of patients carrying each allele, individually, were ABPA subjects, suggesting an additive effect. How these SNPs affect SP-A has not yet been elucidated, but the collagen region spanning both SNPs has been shown to associate with receptors of alveolar macrophages [74], which are important in protecting against *Aspergillus* colonization [22]. It is theorized that changes in conformation or affinity of SP-A2 may decrease these interactions and compromise host defense.

3.5. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene Mutations. Because ABPA is found in highest incidence among atopic patients with CF, Miller et al. [75] examined mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) in subjects without CF (Table 1). Their group reported that mutations were present at a higher frequency in asthmatic patients who developed

ABPA, 6 of 21 (28.5%), versus control asthmatics, 2 of 43 (4.6%). These ABPA patients were heterozygous for the mutations (1 patient was compound heterozygote and reclassified as atypical CF), did not have a clinical diagnosis of CF, and had sweat chlorides <60 mEq/L. Although the abnormal airway mucus in CF is thought to be a susceptibility factor for ABPA due to enhanced trapping of *Aspergillus* spores, it is unclear what effect heterozygous CFTR mutations may have on mucus quality in asthmatic airways.

3.6. Toll-Like Receptor (TLR) Polymorphisms. Carvalho et al. [76] examined Toll-like receptor (*TLR*) polymorphisms of *TLR2*, *TLR4,* and *TLR9* in cavitary pulmonary aspergillosis (CCPA) and severe asthma associated with fungal sensitization (SAFS). TLR-4 is among the major receptors for *Aspergillus* hyphae and plays an important part in innate host defense as TLR-4-deficient mice have increased susceptibility to invasive aspergillosis [77]. In CCPA patients, there was significantly increased frequency of the G allele of *TLR4* on asp299gly. ABPA patients had increased frequency of allele C for the *TLR9* T-1237C polymorphism compared to control patients. However, in SAFS patients who are predominantly *Aspergillus* sensitive, there was no association of polymorphisms of *TLR2*, *TLR4*, or *TLR9*. TLR-9 is a receptor that recognizes CpG motifs prevalent in bacterial and viral DNA. *Aspergillus* hyphae and conidia do signal through TLR-9 on murine neutrophils [78]. TLR-9-deficient mice demonstrate greater conidial and hyphal damage. In addition, Lazarus et al. [79] reported that TLR9 polymorphisms have been associated with increased risk of asthma. Novak et al. [80] reported that the *TLR9* C allele of T-1237C decreases expression. Thus, decreased TLR-9 protective function may be an underlying susceptibility in the development of ABPA.

4. Conclusions

The prognosis of ABPA is good if the disease is detected early and treatment started promptly. It is important that the diagnosis is made and treatment commenced before there is permanent lung damage from bronchiectasis. In such patients, there should be no progression of the disease, although relapses can occur many years later, and long-term followup is recommended. In children with CF, the relapses seem to be more frequent than they are in patients with asthma, and careful surveillance is necessary to ensure resolution of the disease process. In some CF patients, it is difficult to wean the steroids without an increase in symptoms, such as dyspnea and wheezing, whether this is due to the underlying CF lung disease or due to patients going from stage II to stage III ABPA on withdrawal of steroids is unclear. Adjunctive treatment with antifungal therapy to *Aspergillus* should be considered. Symptoms are not a reliable guide to therapy; therefore, it is important to reevaluate the chest radiograph and the serum IgE at regular intervals until a long-term remission is established.

ABPA occurs with a worldwide distribution in a significant number of patients with CF and less frequently in those with asthma. Early diagnosis and treatment are essential in preventing end-stage progression. The development of ABPA is probably the combination of many genetic susceptibility factors, gene-gene interactions, and environmental exposure which work together. Understanding of the genetic risks and immunopathogenesis of ABPA hopefully will lead to improved early diagnosis and improved treatment of ABPA.

Abbreviations

Af:	*Aspergillusfumigatus*
Asp fx:	*Aspergillus fumigatus* proteins
APC:	Antigen presenting cell
MBP:	Major basic protein
ECP:	Eosinophil cationic protein
EDN:	Eosinophil derived neurotoxin
VLA:	Very late activation antigen
VCAM:	Vascular cell adhesion molecule
CxCR and CCR:	Chemokines receptors
MCP:	Monocyte chemotactic protein
sCD23:	Soluble CD23
cyst-LT:	Cysteinyl leukotriene
ABPA:	Allergic bronchopulmonary aspergillosis
CFTR:	Cystic fibrosis transmembrane conductance regulator
IL-4RA:	IL-4 receptor alpha chain
TARC:	Thymus and activation-regulated chemokine
SP-A2:	Surfactant protein A2 polymorphisms.

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