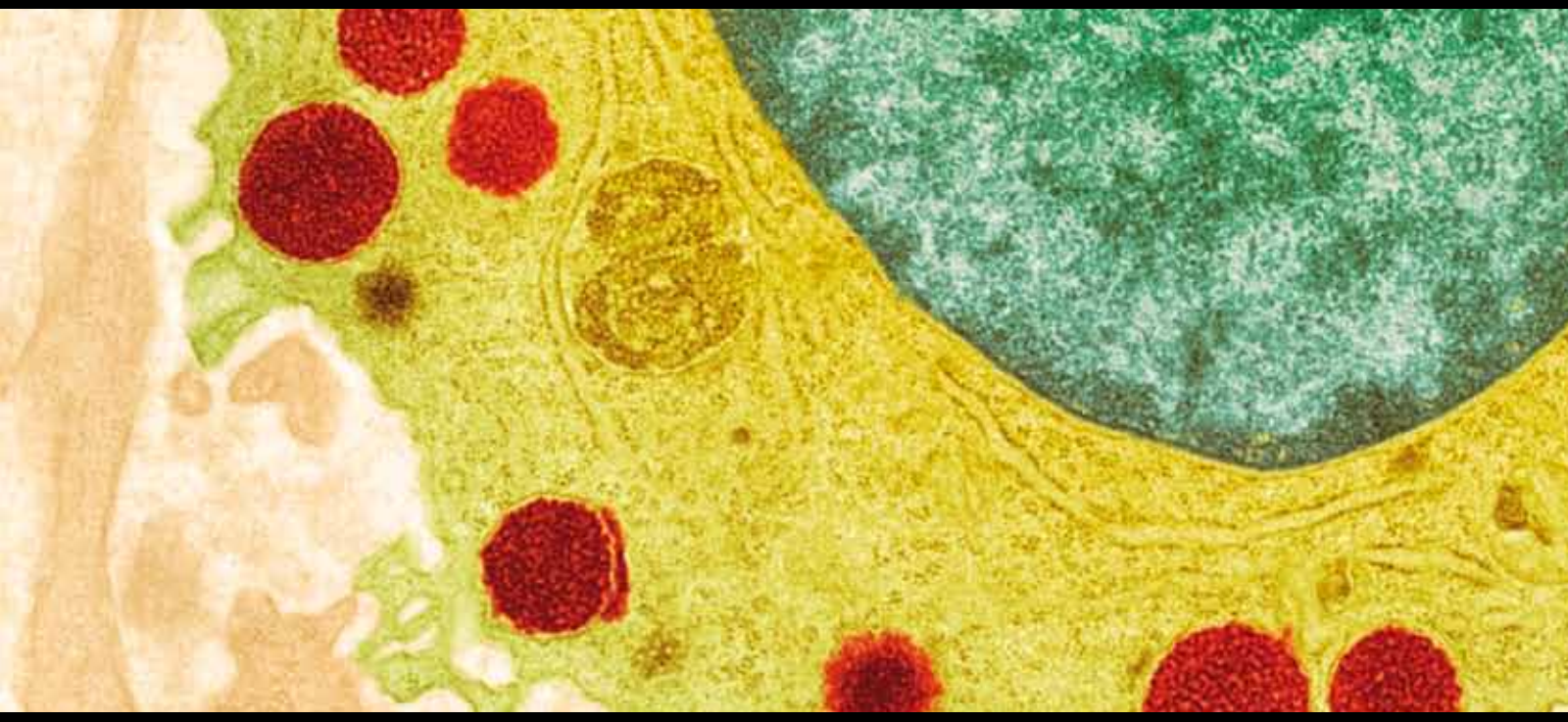
Journal of Allergy

Beyond the Immune System: The Role of Resident Cells in Asthma and COPD

Guest Editors: Peter Borger, Brian Oliver, Irene Heijink, and Georgia Hardavella



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Editorial Board

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Editorial

Beyond the Immune System: The Role of Resident Cells in Asthma and COPD

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Asthma and chronic obstructive pulmonary disease (COPD) are the two most prominent chronic inflammatory lung diseases, and their prevalence is still increasing. Although both diseases are associated with genetic and environmental factors, their precise etiologies are still unclear. Recent advances in biology and medicine have introduced new technologies to study the genetics and function of resident cells of the lungs, and the data demonstrate a much broader range of impact than previously envisaged. The function of resident cells of the lung may be crucial to fully understand the pathology of asthma and COPD. Knowledge and understanding of resident cells will lead to the development of improved animal models, more successful therapies, novel tools to characterize asthma, and COPD and provide better care to patients. In our present special issue, several outstanding research groups present their original research articles that will stimulate the continuing efforts to understand the molecular pathology underlying asthma and COPD, the development of strategies to treat these conditions, and the evaluation of outcomes. Moreover, several important novel insights on the role of resident cells in asthma and COPD are being reviewed in this special issue.

Y. Matsumura reviews the proteinase activity of many common allergens and shows their interaction with lung epithelial cells; they induce disruption of the tight junctions between epithelial cells, activate the protease-activated receptor-2, and lead to the production of thymic stromal lymphopoietin. Hence, allergen source-derived proteases are a potentially critical factor in the development of allergic sensitization and appear to be strongly associated with heightened allergenicity. S. Kolahian and R. Gosens describe a novel role of the parasympathetic neurotransmitter acetylcholine in the regulation of airway remodeling and inflammation in respiratory disease. Recent data have indicateed that nonneuronal cells, including smooth muscle cells, secrete acetylcholine and express receptors for acetylcholine. S. Kolahian and R. Gosens focus on the role of acetylcholine in smooth muscle cell function and review data to show that the activation of acetylcholine receptors in these cells can lead to proliferation, production of growth factors, inflammatory mediators, and deposition of extra-cellular matrix proteins. Moreover, it can lead to increased smooth muscle mass, contractility, mucus gland remodeling and airway inflammation *in vivo* in a guinea pig model of asthma. Similarly, tiotropium studies show that acetylcholine may contribute to cigarette smoke-induced neutrophilic airway inflammation in a murine model of COPD. In addition to the new role of acetylcholine, N. Miglino et al. present data on another novel target to reduce bronchial smooth muscle remodeling in their original research article. They show that calreticulin, which has been described as a negative regulator of C/EBPα [1], is also able to reduce airway smooth muscle (ASM) cell proliferation and may thus provide beneficial effects on airway remodeling. This pathway was insensitive to corticosteroids, which may explain why airway remodeling in asthma patients is refractory to corticosteroid therapy [2]. In

particular subpopulations of asthma patients do not respond well to the current therapy available for asthma. To identify more effective treatments, it is of importance to unlock the underlying mechanisms and understand the genetic background. A. Faiz and K. Burgess review the history of expression microarray technologies, that is, genome-wide association studies (GWASs)/locus fine mapping, gene candidate approaches, and gene expression studies (gene-expression microarrays, including the Gene-Chip), and their contribution to increase our understanding of asthma pathology. For instance, GWASs have been essential in the discovery of many asthma-associated genes, including disintegrin and metalloproteinase domain-containing protein 33 (ADAM33). In addition, the 3′ expression arrays have shown that treatment with IL-13 caused deregulation of a number of asthma-related genes in different cell types of the airway, including smooth muscle cells and airway epithelium.

The ASM cell has been recognized as a critical effecter cell in the pathophysiology of asthma for almost a century. The interplay between the smooth muscle and the pathophysiology of asthma is reviewed by A. Ozier and colleagues. Specifically they discuss the mechanisms driving airway hyperresponsiveness in asthma, such as mediator release, altered excitation/contraction coupling, and the role of airway smooth muscle in bronchial inflammation and remodeling. T. Chiba et al. prove a compelling review on the role of the arylhydrocarbon receptor (AhR), a nuclear receptor which responds to dioxins and dioxin-like compounds in cigarette smoke and environmental pollutants. In their review they discuss the role of AhR in asthma and COPD, focusing upon how AhR modulates the immunological responses in allergic and inflammatory diseases such as bronchitis, asthma, and chronic obstructive pulmonary disease (COPD) and the crosstalk of AhR signaling with other ligand-activated transcription factors such as peroxisome proliferator-activated receptors (PPARs). In addition to the role of mesenchymal cells in innate immunity, mesenchymal cells also have the ability to direct and in-turn can be influenced by the adaptive immune system. One novel example of this interaction is the role of immunoglobulin receptors found upon the surface of mesenchymal cells. In this issue, N. Roth reviews the role of immunoglobulin receptors upon mesenchymal cell function and importantly addresses some of the basic questions in this field: why do tissue-forming cells express immunoglobulin receptors and do tissue-forming cells process immunoglobulin receptor-bound particles?

Several drugs are currently in use targeting the inflammatory component of the asthmatic disorder, including steroids and beta-mimetics [3]. It is well-established that rhinovirus (RV)-induced asthma exacerbations account for high asthma-related health costs and morbidity [4]. D. van Ly and coworkers studied the molecular mechanism underlying this pathology involving RV-induced nuclear factor kappa B (NF-κB)-dependent inflammation. To establish the role of NF-κB inhibitors in RV-induced IL-6 and IL-8 and RV replication, they used pharmacological inhibitors of NF-κB, and steroids and/or β2 agonists were used as comparison. Their data suggest that targeting NF-κB alone is unlikely to be an effective treatment compared to current asthma therapeutics. Q. Ge and coworkers examined the effect of combined corticosteroids and long acting beta2-agonists on *in vitro* extracellular matrix protein production by TGF-stimulated airway smooth muscle (ASM) cells present in isolated bronchial rings of nonasthmatic individuals. They conclude that current combination asthma therapies are unable to prevent or reverse remodeling events that are regulated by ASM cells. Current asthma therapies have also been in the spotlight of J. C. Allen et al. who investigated corticosteroid insensitivity *in vitro* and were the first to examine the effect of dexamethasone on the mitogen PDGF-BB-induced cyclin D1 upregulation in ASM cells from both nonasthmatics and asthmatics. They showed that cyclin D1 mRNA and protein up-regulation in cells from asthmatic patients cannot be totally inhibited. These results agree with earlier evidence that corticosteroid-inhibited proliferation occurs only in ASM cells from nonasthmatics [5] and suggest that there are corticosteroid insensitive proliferative pathways in asthmatics that need to be further investigated towards new efficacious antiremodeling strategies.

A. Soltani and coworkers have had substantial experience in using Collagen IV antibody as a blood vessel marker in bronchial biopsies [6]. Therefore, they compared the utility of anti-Collagen IV and anti-Factor VIII antibodies as markers for blood vessels in bronchial biopsies from COPD versus normal subjects and investigated the differences in the vessels' profiles that they stained. They showed that anti-Collagen IV antibody tends to stain more vessels in the Rbm and bigger vessels overall in both the Rbm and LP, while anti-Factor VIII antibody stains relatively smaller vessels. A novel finding of this study was the increased leakiness of the vessels in the Rbm in current smokers with COPD which is ripe for speculation and further study and could be factored into the concepts of the pathogenesis of smoking-related airway disease pathophysiology. The complex pathophysiology of COPD and asthma can be clarified by the use of recent advances in CT imaging. G. Dournes et al. present a thorough yet concise review of quantitative measurements of airway wall and lung parenchyma in both COPD and asthma by using CT. Quantification of airway wall and lung parenchyma has demonstrated strong correlations with clinical, functional, and pathological features in humans or animal models.

Taken together, this special issue provides a focused overview and state-of-the-art science related to the role and function of resident cells of the lung, and how they may be involved in developing and maintaining asthma and COPD pathologies.

Peter Borger
Brian Oliver
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Research Article

Reticular Basement Membrane Vessels Are Increased in COPD Bronchial Mucosa by Both Factor VIII and Collagen IV Immunostaining and Are Hyperpermeable

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Background and Objective. Using Collagen IV staining, we have previously reported that the reticular basement membrane (Rbm) is hypervascular and the lamina propria (LP) is hypovascular in COPD airways. This study compared Collagen IV staining with vessels marked with anti-Factor VIII and examined vessel permeability in bronchial biopsies from COPD and normal subjects using albumin staining. *Results*. Anti-Collagen IV antibody detected more vessels in the Rbm ($P = 0.002$) and larger vessels in both Rbm ($P < 0.001$) and LP ($P = 0.003$) compared to Factor VIII. COPD airways had more vessels (with greater permeability) in the Rbm ($P = 0.01$) and fewer vessels (with normal permeability) in the LP compared to controls with both Collagen IV and Factor VIII antibodies ($P = 0.04$ and $P = 0.01$). *Conclusion*. Rbm vessels were increased in number and were hyperpermeable in COPD airways. Anti-Collagen IV and anti-Factor VIII antibodies did not uniformly detect the same vessel populations; the first is likely to reflect larger and older vessels with the latter reflecting smaller, younger vessels.

1. Introduction

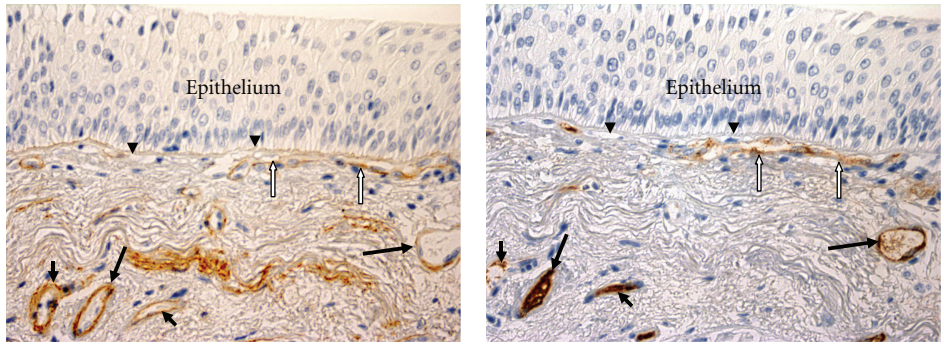
Angiogenesis is under vigorous study in many diseases including chronic inflammation and malignancies. Chronic inflammatory diseases of the airways such as asthma and COPD are no exceptions [1, 2].

For better detection of blood vessels, specific stains are needed as haematoxylin and eosin alone are not specific enough [3]. The most commonly used tissue vessel markers in studies of the respiratory tract have been antibodies against Collagen IV and Factor VIII, CD31 (EN-4) and CD34. Both glycol methacrylate (GMA) processing and paraffin embedding are superior to other methods for investigation of vessels in tissue samples [4].

Factor VIII antigen is produced by endothelial cells and is physiologically involved in platelet aggregation and adhesion [5, 6]. A number of studies have reported Factor VIII antibody as a reliable marker for blood vessel detection [7]. However, it has been reported that Factor VIII antibody also stains megakaryocytes, mesenchymal tissue, and immune cells in addition to endothelial cells [4, 8]. The efficiency of Factor VIII antibody for detecting blood vessels has also been shown to be related to the size of vessels [9, 10].

Our group has had substantial experience in using Collagen IV antibody as a blood vessel marker in bronchial biopsies (BB) [2, 11–14]. Collagen IV antibody delineates endothelial basement membrane [3, 4].

An optimal marker for blood vessels should be specific, independent of pathological changes in tissue (e.g., inflammation, malignancy, hypoxia, ischemia, shearing stress), resistant to the usual methods of tissue fixation and processing, open to detection of a variety of sizes (i.e., large and small) and ages (i.e., old and new) of vessels, and be able to detect different types of vessels, that is, capillary, vein, arteriole, and artery. It has been shown that under both physiological and pathological conditions, endothelial cells modify their antigen presentation [10], and most available histochemical markers do not fully meet these characteristics, but the pros and cons of different immunohistologic antibody systems have not been worked out in any detail.



(a) (b)

Figure 1: Vessels in the bronchial mucosa. Bronchial biopsies taken from the same current-smoking COPD subject, ×400. Vessels (black arrows) are stained with anti-Collagen IV antibody (a) and anti-Factor VIII antibody (b). A large vessel which contacts the reticular basement membrane (Rbm, arrowheads) is indicated by white arrows. The epithelium is thickened, probably because of chronic smoking exposure.

Antibodies to Collagen IV and Factor VIII stain different epitopes and indeed different structures in vessels, and the literature indicates that these markers for immunostaining of vessels do not uniformly detect vessels of different sizes and can vary in efficiency in pathological processes. Therefore, we decided to compare the utility of anti-Collagen IV and anti-Factor VIII antibodies as markers for blood vessels in BB from COPD versus normal subjects and investigate what the differences are in the vessel profiles that they stain. Based on our previous findings [1, 2], we would expect any results in this study to apply generally to smokers as well as COPD.

Permeability of mucosal vessels in asthma has been reported to be increased and to correlate with clinical deterioration [15]. Vascular endothelial growth factor (VEGF) increases vascular permeability to blood water along with proteins [16]. We have reported increased vessel-related VEGF in the Rbm of COPD airways with the changes most marked for current smoking COPD patients [2]. Therefore, it is also reasonable to study vessel leakiness in the Rbm of bronchial wall in current smoking COPD subjects.

2. Material and Methods

This was a *cross-sectional study*. *Subjects* were recruited by advertisement. The study was approved by the *Human Research Ethics Committee (Tasmania) Network,* and all subjects provided written informed consent. Twenty-eight mild to moderate COPD subjects and eight normal nonsmoking controls participated. COPD was diagnosed using GOLD criteria [17]. Subjects with other respiratory diseases including a clinical history of asthma were excluded. All COPD subjects were on short-acting anticholinergic bronchodilators only. *Lung function tests* were performed according to ATS/ERS guidelines [18]. *Fiberoptic bronchoscopies and endobronchial biopsies* were performed as previously described [2]. There were no complications from the procedures.

2.1. Tissue Processing. 2 × paraffin-embedded sections of 3 μm and 50 μm apart mounted on APTS-coated slides were used. Following dewaxing and hydration, sections were subjected to heat retrieval using Dako S1700 for 20 minutes (except Albumin which did not require epitope retrieval), and then endogenous peroxidase was quenched using 3% hydrogen peroxide for 15 minutes. Sections were incubated in primary antibodies for either Von Willebrand factor (Factor VIII-related antigen) (Dako M06160), Type IV collagen (Dako M0785) (both at 1 : 150 for 90 minutes) (Figure 1), or Albumin (Abcam Ab 2406) 1/6000 for 30 minutes at 20 degrees Celsius. For negative controls, matched sequential sections were stained with primary antibody, replaced with a species-appropriate IgG1 isotype, at equivalent dilutions and conditions. A horseradish peroxidase (HRP) conjugated DAKO Envision plus (Dako K4001) reagent was used for secondary antibody binding and DAB PLUS (DAKO K3468) for color resolution (brown). Mayer's haematoxylin counterstain was used to elaborate nuclei. Sections were dehydrated in ethanol, cleared in xylene, and mounted in Permount prior to analysis.

Measurements were performed using a computer-assisted image analysis tool (Image-Pro version 5.1, Media Cybernetics, USA). Pictures of all intact and nonoverlapping areas were taken from each slide, and eight separate fields were chosen randomly for measurements to have on average 3 mm of the Rbm in our measurements as we did in our previous report [2]. The histologist (AS) who performed the measurements was blinded to the diagnoses and order of slides, which had been independently randomly sorted and coded.

Table 1: Demographics.

	Group	
	Control ($n = 8$)	COPD ($n = 28$)
Age*, median (interquartile range), years	54 (9)	61 (10)
Gender†, female (number)	2	9
Pack-year smoking history, median (interquartile range)	0	47 (23)
FEV1/FVC%¶, median (interquartile range)	79 (17)	57 (16)

*$P = 0.1$ (Mann-Whitney test).
†$P = 0.2$ (Fisher's Exact test).
¶$P < 0.001$.

Table 2: Agreement between two methods of vessel staining in both groups together.

	Mean, Collagen IV	Mean, Factor VIII	Mean of differences*	95% LoA†
Number of vessels/mm Rbm	8.5	4.8	+3.7	−10.2, +17.6
Area of vessels/mm Rbm	803	357	+446	−995, +1887
MVS of Rbm vessels, μm^2/number	98	73	+25	−143, +193
Number of vessels/mm^2 LP	313	356	−43	−333, +247
Area of vessels $\mu m^2/\mu m^2$ of LP × 100	5.7	4.2	+1.5	−4.4, +7.4
MVS of LP vessels, μm^2/number	212	133	+79	−184, +342

* Calculated as measurements with Collagen IV minus measurements with Factor VIII antibody.
† LoA: limits of agreement (mean ±2 standard deviation).

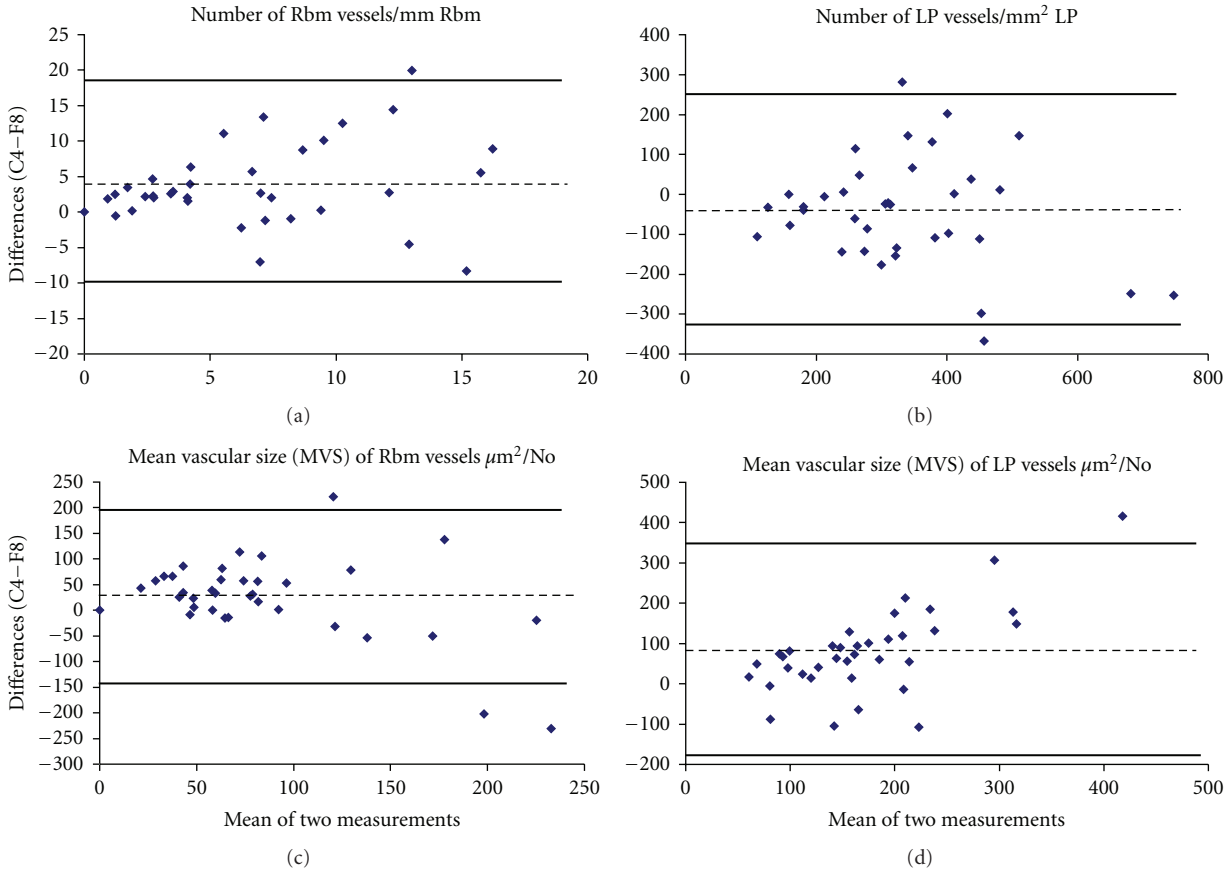


Figure 2: Bland and Altman plots for number of vessels and mean vascular size (MVS) in the reticular basement membrane (Rbm) and lamina propria (LP) in all study subjects together. Bland and Altman plots illustrate agreement between the two methods of vessel staining with anti-Factor VIII and anti-Collagen IV antibodies. Values on the *X* axis represent means of the two measurements; values on the *Y* axis indicate measurements with Collagen IV staining minus measurements with Factor VIII staining (C4-F8). The broken line represents the mean difference. Bold lines indicate Limits of agreements (mean of differences ±2 standard deviations). C4: anti-Collagen IV; F8: anti-Factor VIII.

To be constant, we used the same methods for measurements so as to be able to compare the results of this current study with our previous report [2]. Vessels in the reticular basement membrane (Rbm) and down to a depth of 150 μm of the subepithelial lamina propria (LP) from the antilumenal margin of the Rbm were measured separately. Only well-formed cylindrical or tubular structures that were stained with immunostaining antibodies were measured as vessels to avoid including nonvascular cells in analyses (Figure 1). Number and cross-sectional area of vessels were measured. These data were normalized by dividing by the length of the Rbm or dividing by the surface area of the LP examined. Mean vascular size (MVS) was calculated as total vascular area/number of vessels. The area of the LP excluded mucous glands and muscle.

For vessel permeability, using Image-Pro 5.1 again, the percentage of compartment tissue area stained for albumin was measured separately in the Rbm and LP in current smoking COPD and normal controls and the results expressed as a percentage (μm^2 of tissue stained/μm^2 tissue

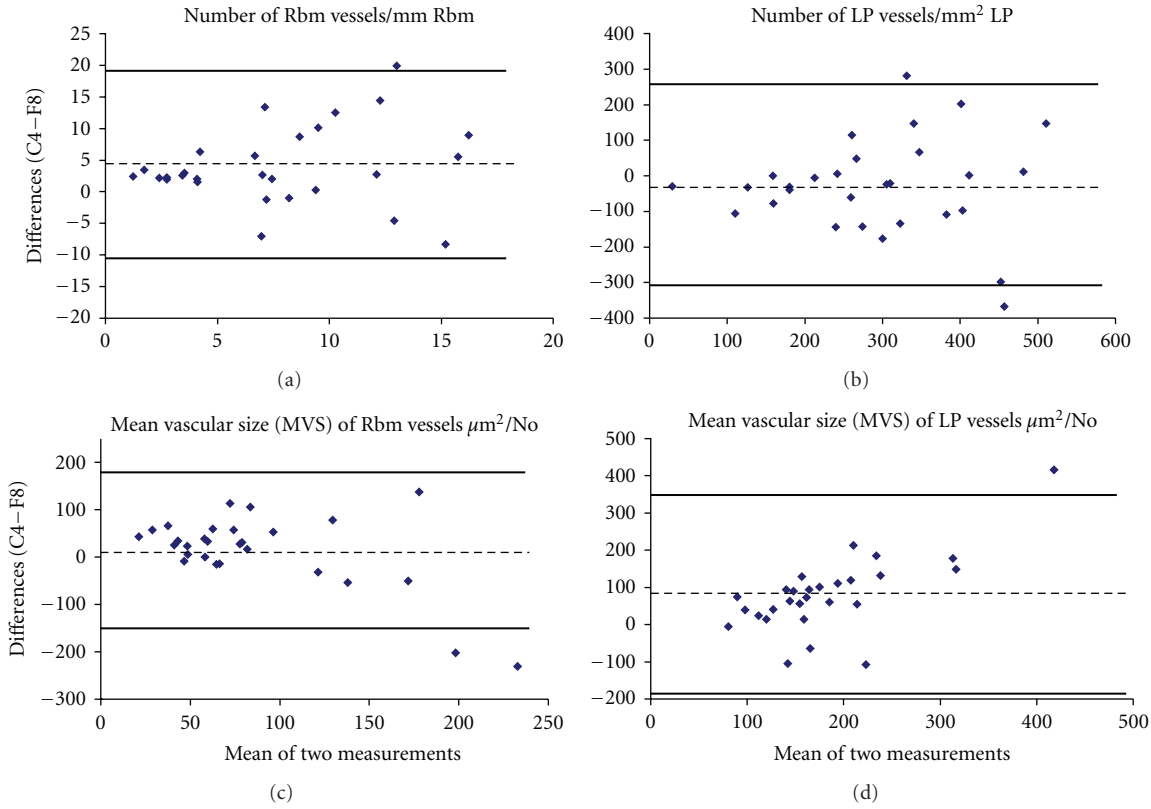


Figure 3: Bland and Altman plots for number of vessels and mean vascular size (MVS) in the reticular basement membrane (Rbm) and lamina propria (LP) in COPD subjects. Number of vessels is expressed as No/mm Rbm and No/mm^2 LP. MVS is presented as area μm^2/No. For MVS in the LP, the agreement between the two methods was best when vessels were relatively smaller and the differences increased as MVS increased, with anti-Collagen IV showing larger MVS than anti-Factor VIII. Description of the measurements and abbreviations are presented in Figure 2.

examined ×100). Finally, the percentage area of perivascular albumin staining was measured within the 10 μm perimeter around vessels (to avoid overlapping of areas) in both the Rbm and LP. We have previously successfully used this method to measure vascular permeability in asthmatic airways [15].

2.2. Statistical Analyses. The data from the two methods of vessel staining were tested for agreement using the method reported by Bland and Altman [19]. Very briefly, by this latter method, the means of the two measurements are plotted against the differences between the two measurements. The 95% limits of agreement (LoA = mean of differences ±2 standard deviations (SD) of differences) were calculated for every measurement.

For comparison of means between two groups or between two methods of staining, Student's t-test was used for variables with normal distribution and the Mann-Whitney test for nonnormally distributed variables. Fisher's Exact test was used to compare gender distribution between two groups. All continuous data were presented as median (interquartile range), except for the data that are included in agreement between the two methods of vessel staining which were presented as mean ±2 standard deviations. P value less than 0.05 was considered as significant. SPSS 16.0 was used for statistical analyses. Pearson's or Spearman tests were used to test correlations for normally and nonnormally distributed variables, respectively. SPSS 16.0 was used for statistical analyses.

3. Results

Thirty-six subjects participated in the study. Table 1 summarizes the demographics. The 28 COPD subjects were balanced between 15 current smokers and 13 ex-smokers. There were no significant differences between groups in age or gender.

3.1. Collagen IV versus Factor VIII. The most important characteristics of agreement between the two methods of vessel staining are summarized in Table 2. Bland and Altman plots show that anti-Collagen IV antibody detected higher number of vessels and larger MVS in the Rbm and lower number of vessels but again larger MVS in the LP compared with anti-Factor VIII antibody in all study groups (Figure 2). This was also the case for the COPD group analyzed separately (Figure 3). For Bland and Altman plots, values on the Y axis in Figures 2 and 3 were calculated as measurements with

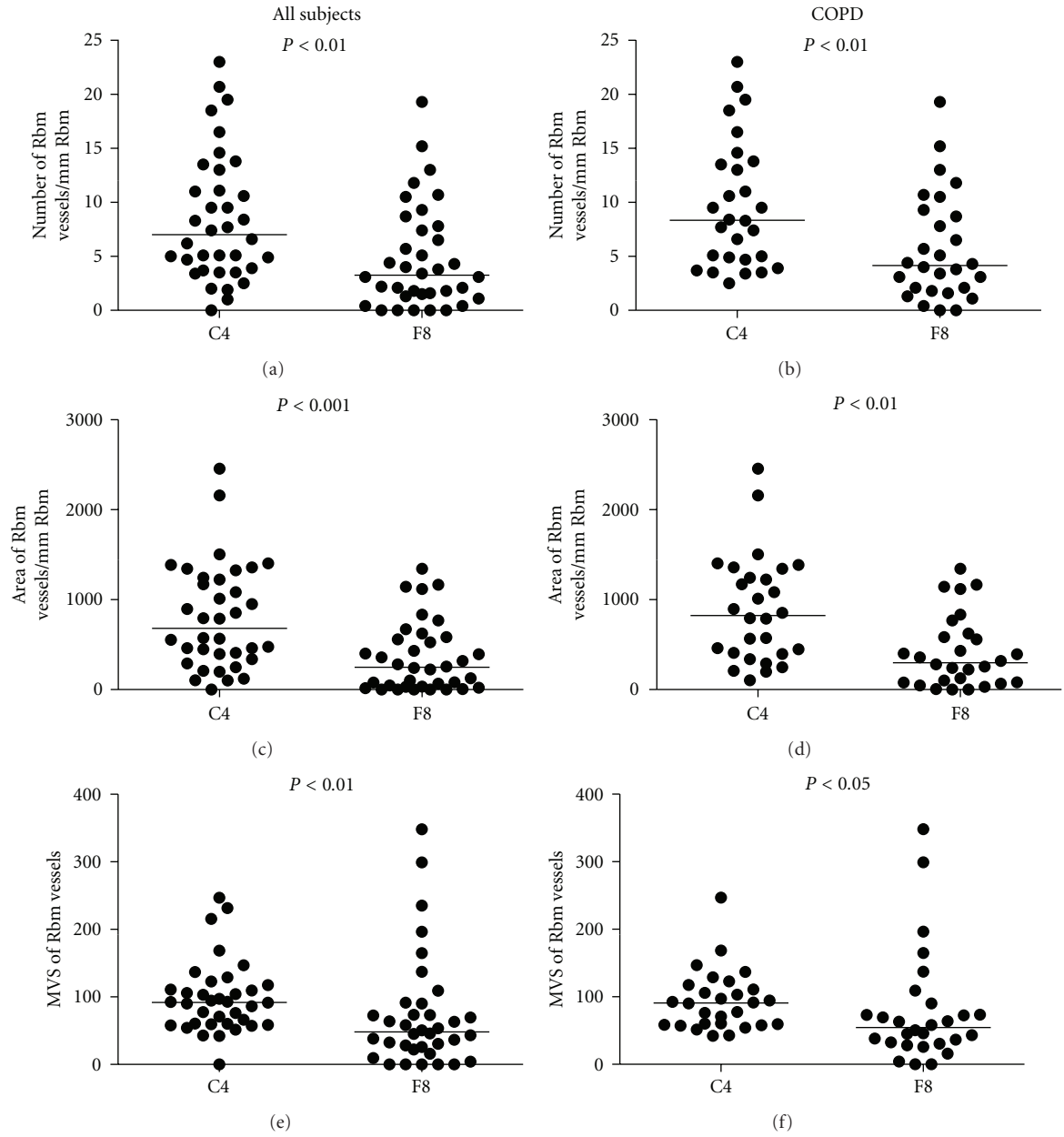


Figure 4: Comparison of the number, area, and MVS of vessels in the reticular basement membrane (Rbm). In both COPD and normal groups, Collagen IV staining outlines more vessels of larger caliber, than Factor VIII staining. MVS: mean vascular size (area μm^2/No); C4: anti-Collagen IV antibody; F8: anti-Factor VIII antibody.

Collagen IV staining minus measurements with Factor VIII staining.

Comparing the means of the absolute number, area and MVS of Rbm vessels stained by the two immunostaining methods confirmed our results with the Bland and Altman plots; there were significantly greater number, area, and MVS of vessels with anti-Collagen IV antibody than with anti-Factor VIII antibody both when all subjects were tested together and when the COPD group was tested alone (Figure 4). In the LP, comparison of the means showed that the differences between two methods of blood vessel staining were significant for area and MVS but not for the number of vessels (Figure 5).

3.2. COPD versus Normal Controls. When COPD subjects were compared to controls, COPD had significantly more vessels in the Rbm and fewer vessels in the LP with both Collagen IV and Factor VIII antibody staining (Figures 6 and 7).

Significantly, more tissue was stained for albumin in the Rbm in current smoker COPD than controls (median (interquartile range), $\mu m^2/\mu m^2$ presented as percent, 0.37% (1.68) versus 0.00% (0.20), $P = 0.02$). But perivascular

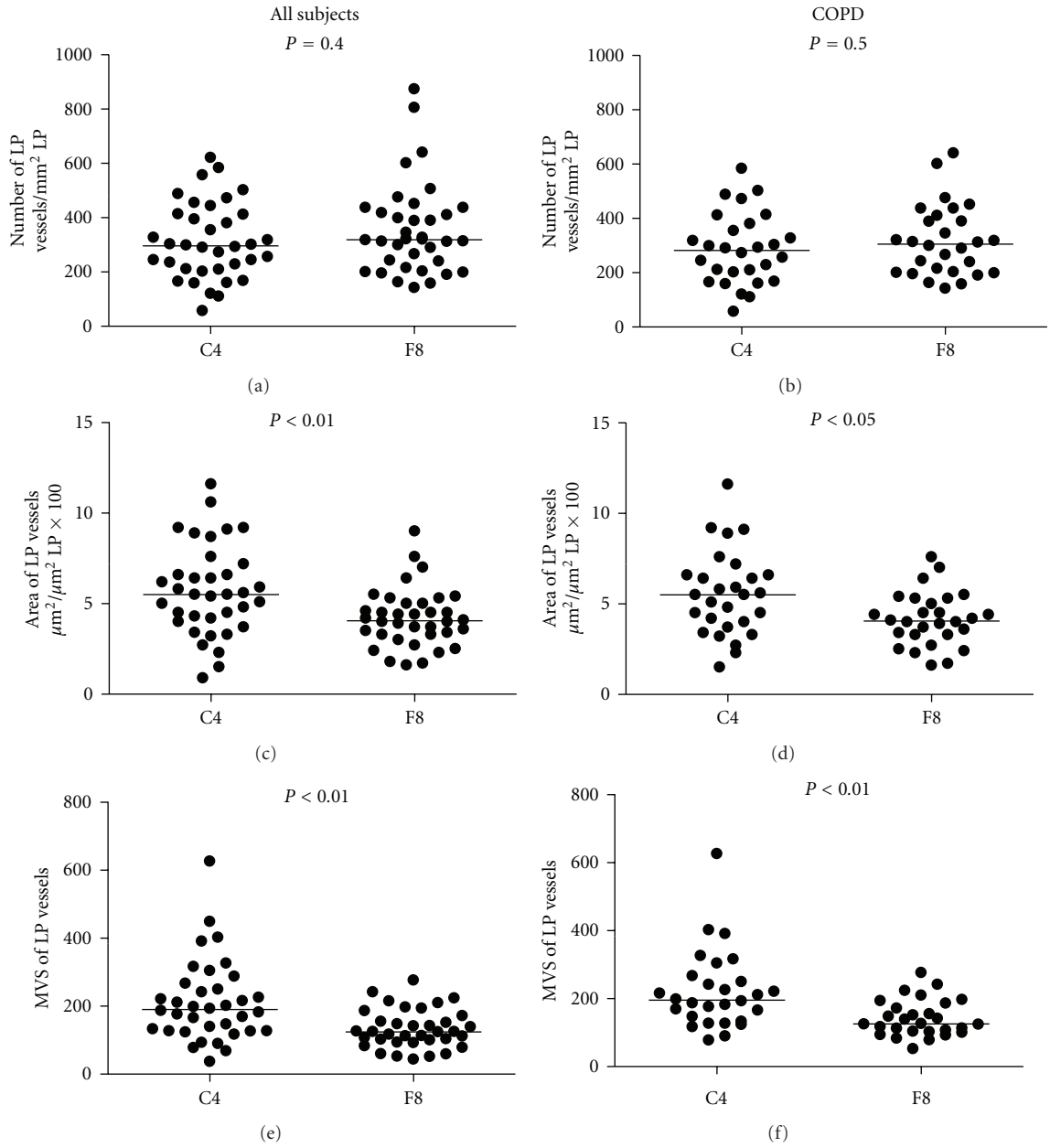


Figure 5: Comparison of the number, area, and MVS of vessels in the lamina propria (LP). In both COPD and normal groups, Collagen IV staining outlines bigger vessels but not more vessels. MVS: mean vascular size (area μm^2/No); C4: anti-Collagen IV antibody; F8: anti-Factor VIII antibody.

albumin in the Rbm or LP and albumin staining in the LP were not significantly different between two groups.

3.3. Correlations. Number of Rbm vessels stained with anti-Factor VIII antibody correlated negatively with forced vital capacity (FVC) only in ex-smokers with COPD (Spearman $r = -0.8$, $P = 0.002$). Otherwise, there were no correlations between our anatomical findings and lung function parameters. We did not find any suggestion of a relationship between either age or pack-years smoking and vascular or permeability changes in the COPD group.

4. Discussion

This study showed that anti-Collagen IV antibody tends to stain more vessels in the Rbm and bigger vessels overall in both the Rbm and LP, while anti-Factor VIII antibody stains relatively smaller vessels. It has previously been shown that vessel markers can have different sensitivity and specificity in detecting vessels in normal versus abnormal conditions. For example, factors such as genetic diversity in endothelial cells, hypoxemia, age, and shearing stresses have effects on the expression of Factor VIII protein [10, 20, 21]. The sensitivity and specificity of these markers are also related to the size

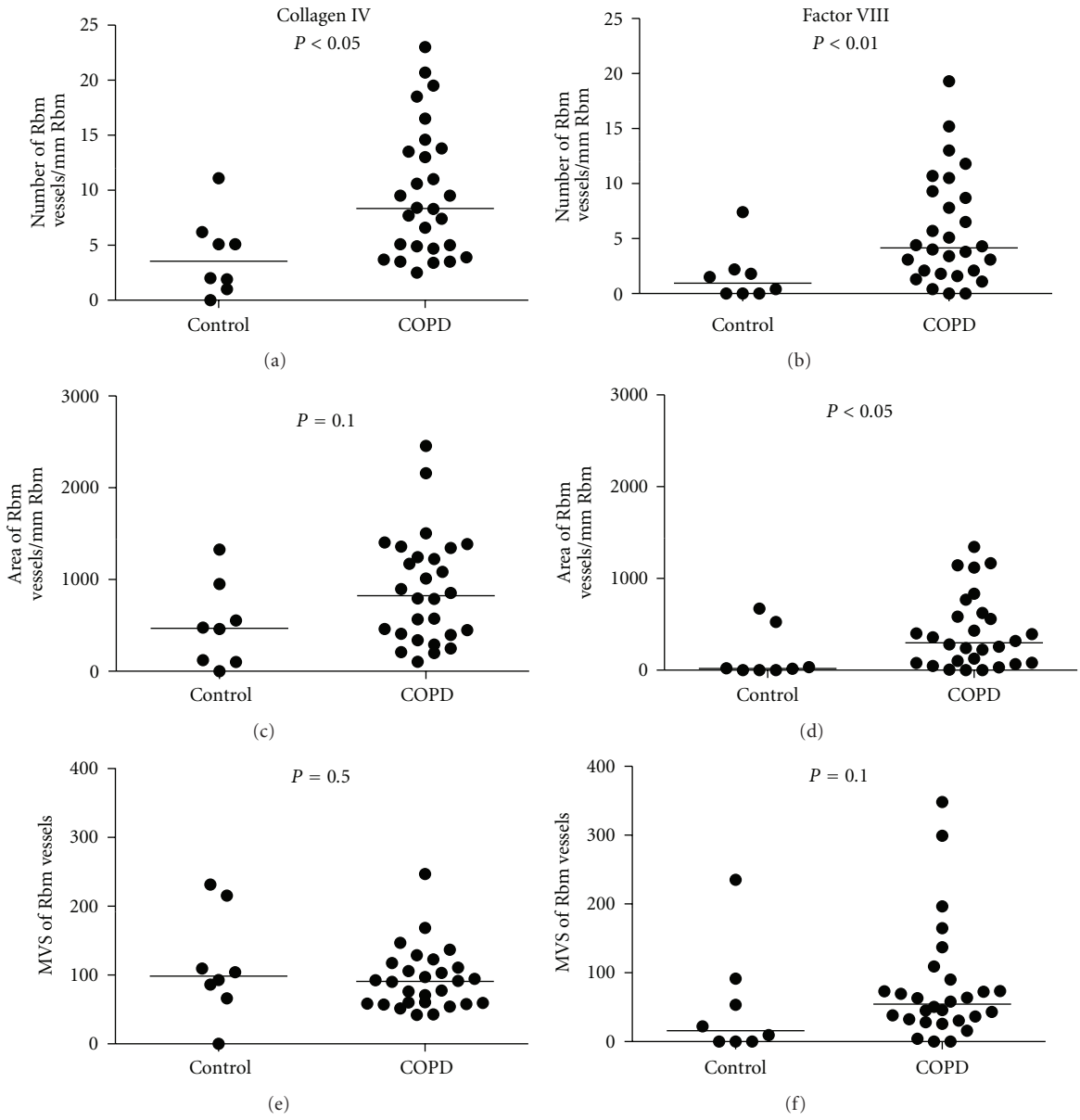


Figure 6: Comparison of reticular basement membrane (Rbm) vessels in COPD and normal control groups. COPD subjects have more vessels with both methods of staining. MVS: mean vascular size (area μm^2/No).

of vessels [9, 22]. Indeed, our data are consistent with a previous report showing that staining for Factor VIII cannot detect larger-sized vessels as accurately as smaller ones in invasive breast cancer [9]. But this type of differentiation has not previously been attempted with airway wall samples; although our previous work did show that there was a shift to greater number and smaller vessels in the LP in asthma [14].

Anti-Collagen IV antibody consistently detected larger vessels in the Rbm than Factor VIII antibody. In the LP, the agreement between the two methods was best when vessels were relatively smaller, so that as the MVS increased, there were increasing differences between the two methods, with anti-Collagen IV again demonstrating larger MVS than anti-Factor VIII (Figures 2 and 3). The literature would suggest that smaller vessels are likely to be disproportionately newer vessels, while larger vessels are likely to be older and more mature or even "post-mature" ghost vessels [23–25]. An in vivo study on mice showed that new vessels in airways, formed under angiogenic stimulation by VEGF, had detectable pericytes and basement membrane by day 7. When the newly formed vessels were deprived of VEGF, firstly the flow of blood stopped, followed by death and then fragmentation of endothelial cells, and finally apoptosis of pericytes. However, a basement membrane sleeve from the whole structure remained for some time [23]. This emphasizes that different markers will demonstrate vessels better or worse depending on their age, maturity, and growth factor environment. We would propose that in conditions

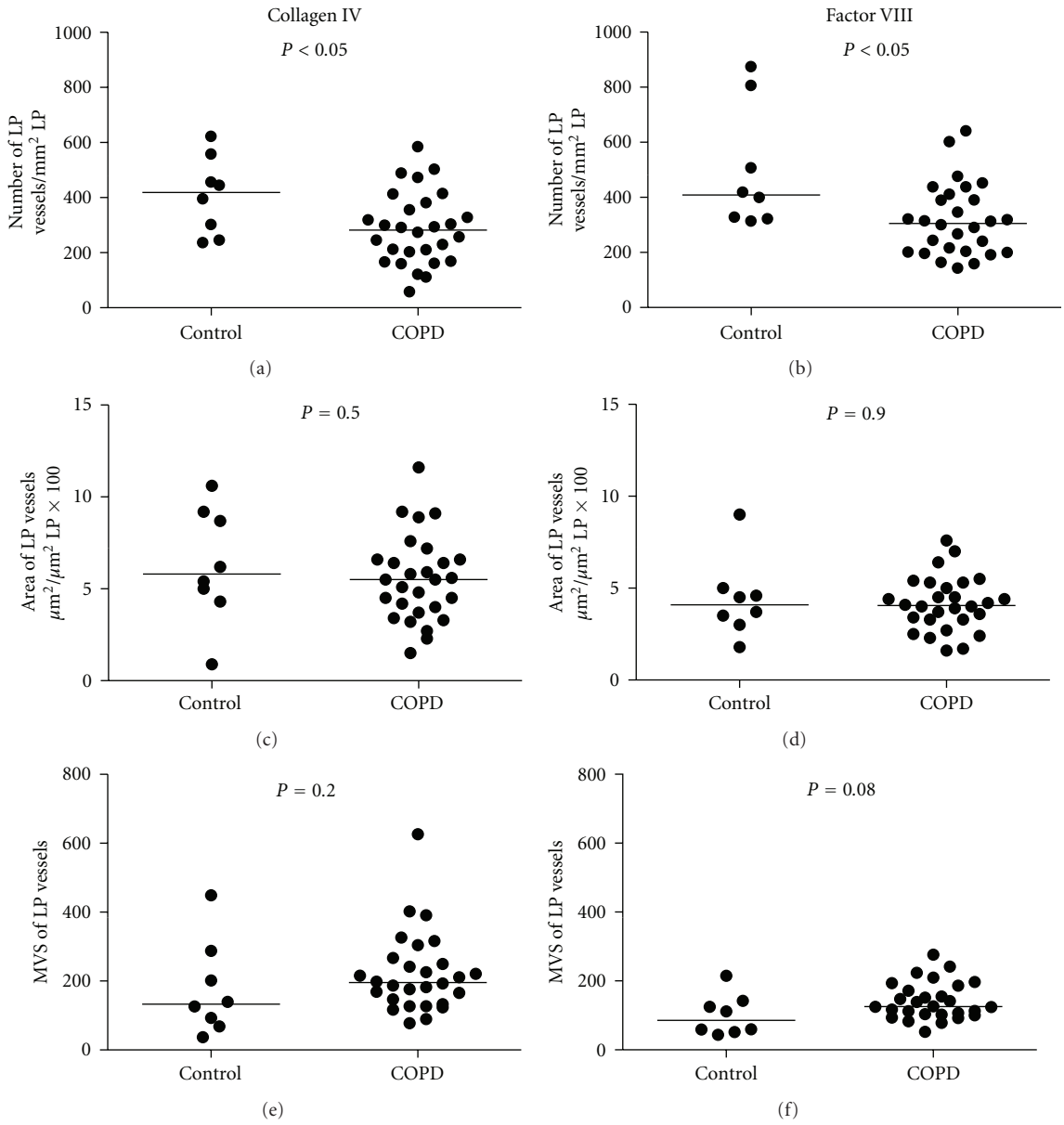


Figure 7: Comparison of lamina propria (LP) vessels in COPD and normal controls. Vessel number is decreased in COPD with both methods. MVS: mean vascular size (area μm^2/No).

where we expect active new vessel formation, such as active asthma or malignancies, anti-Factor VIII antibody may detect these newer vessels better than anti-Collagen IV antibody. This could also be potentially useful in evaluating the effects of treatments on vascular regression, for example, the effects of inhaled corticosteroids on vessels in the airways [13]. In contrast, vessels which are larger and probably older are more effectively detected when stained with anti-Collagen IV antibody rather than with anti-Factor VIII antibody. On this basis, however, we cannot explain why the number of Rbm vessels is significantly increased with anti-Collagen IV compared to anti-Factor VIII antibody; we rather expected the data to be the other way round, that is, more new and younger vessels. We suggest that this could be the result of a high number of aged vessels with well-formed endothelial basement membrane where the endothelium has sufficiently matured to lose its Factor VIII antigens.

A novel and especially interesting finding of this study was increased leakiness of the vessels in the Rbm in current smoking COPD. However, in contrast to our hypothesis, we did not find a correlation between albumin staining in the Rbm and vessel-associated VEGF (data obtained in our previous study [2]). Leak of plasma and its protein material could provide an appropriate environment for angiogenesis by extravasation of fibrinogen and formation of a fibrin gel. Endothelial cells and other mesenchymal cells can easily settle and grow in this environment [26]. Therefore, this finding is compatible with the hypervascularity of the Rbm

we have demonstrated in COPD. The consequence of having leaky vessels just below the epithelium in COPD is ripe for speculation and further study, but, at the very least, it may contribute to fluid and protein flux into the airway lumen and add to the mucus volume and constituents. This has never previously factored into the concepts of the pathogenesis of smoking-related airway disease pathophysiology.

Both Collagen IV and Factor VIII immunostaining of bronchial biopsies in this study confirmed our previous findings of hypervascularity of the Rbm and hypovascularity of the LP in the COPD group compared to the control group [2]. The negative correlation between FVC (likely to reflect predominantly small airway narrowing) and Rbm vessels emphasizes the potential functionally detrimental effect of vascular remodeling in the bronchial mucosa in COPD.

5. Conclusions

Larger and probably more mature vessels were detected better by anti-Collagen IV antibody, while smaller and probably newer vessels were detected better by anti-Factor VIII antibody. Increased permeability of vessels in the Rbm of current smoking COPD subjects could be related to the hypervascularity of this compartment and add to its potential functional significance. Both anti-Factor VIII and anti-Collagen IV antibodies confirmed hypervascularity of the Rbm and hypovascularity of the LP in the COPD bronchial mucosa compared to normal.

Conflict of Interests

None of the authors has any competing interests to declare.

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Review Article

Role of Allergen Source-Derived Proteases in Sensitization via Airway Epithelial Cells

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Protease activity is a characteristic common to many allergens. Allergen source-derived proteases interact with lung epithelial cells, which are now thought to play vital roles in both innate and adaptive immune responses. Allergen source-derived proteases act on airway epithelial cells to induce disruption of the tight junctions between epithelial cells, activation of protease-activated receptor-2, and the production of thymic stromal lymphopoietin. These facilitate allergen delivery across epithelial layers and enhance allergenicity or directly activate the immune system through a nonallergic mechanism. Furthermore, they cleave regulatory cell surface molecules involved in allergic reactions. Thus, allergen source-derived proteases are a potentially critical factor in the development of allergic sensitization and appear to be strongly associated with heightened allergenicity.

1. Introduction

Asthma is regarded as an inflammatory disorder of the airways and has generally been recognized as being driven by T helper 2- (Th2-) skewed Th cell differentiation. Th2-driven cytokines, interleukin (IL)-4 and IL-13, trigger B cells to synthesize IgE, while IL-5 plays a role in eosinophil maturation and survival, and IL-13 regulates airway hyperresponsiveness and mucus hyperplasia.

Epithelial cells clearly play important roles in the initiation of Th2 cell responses to allergens. The epithelial cell layer also acts as a molecular sieve that excludes invaders and plays an important role in homeostasis. Barrier function disorder due to filaggrin (FLG) mutations is critical in the pathogenesis of atopic dermatitis [1]. Although FLG is not expressed in the lower airway respiratory epithelium [2], barrier function of the airway epithelium is impaired in asthma, showing shared common underlying pathogenic mechanisms.

Taking these findings together, asthma can be viewed as a disease of both excessive activation and impairment of airway epithelial barrier function [3–5].

Sources of allergens, such as pollen, house dust mites (HDMs), cockroaches, and fungi, may produce or contain proteases and thereby activate and disrupt the epithelial barrier, causing greater sensitization.

This paper focuses on the importance of allergen source-derived proteases as a factor contributing to primary sensitization to allergens and to exacerbation of allergic disorders secondary to impaired epithelial barrier function.

2. Allergen Source-Derived Proteases

Environmental exposure to allergens is an important determinant of the prevalence of asthma. Allergen source-derived proteases act not only as allergens, but also as promoters of allergenicity.

2.1. Pollens. Pollen allergens have protease activity. The pollens of Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtusa*), and Rocky Mountain juniper (*Juniperus scopulorum*) contain serine protease activity [6]. An aspartic protease was also recently identified in Japanese cedar pollen allergen [7]. In grass, two serine proteases from short ragweed (*Ambrosia artemisiifolia*) pollen have been purified and characterized [8, 9]. Kentucky blue grass (*Poa pratensis*), rye grass (*Lolium perenne*), and Bermuda grass (*Cynodon dactylon*), pollen have also been

characterized. These pollens exhibited peptidase activity, which appeared to be from serine proteases, but cystein protease activity was also detected in Kentucky and rye grass pollen [10]. Grass pollen major group 1 allergens are reported to be cysteine proteases [11, 12]. The pollens of white birch (*Betula alba*) and short ragweed contain not only serine but also cysteine protease activity [6, 13].

2.2. HDMs. HDMs produce cysteine and serine proteases. *Dermatophagoides pteronyssinus* 1 (Der p 1) and Der p 3 [14] are cysteine proteases. Der p 6 and Der p 9 are serine proteases [15–17]. Interestingly, Der p 2, which lacks apparent protease activity, is a structural mimic of MD2, a component of the Toll-like receptor-4 (TLR-4) complex, and can reconstitute a TLR4 signaling complex [18], independently of protease effects.

2.3. Cockroaches. American cockroach (*Periplaneta americana*) and German cockroach (*Blattella germanica*) allergen extracts have complex proteolytic activities [19–21]. An approximately 28 kDa trypsin-like serine protease (Per a 10) was purified and characterized from the whole body extract of American cockroaches [22, 23]. Bla g 2, a potent allergen from German cockroaches, has been identified as an aspartic protease [24, 25]. German cockroach extract is rich in proteases and exerts direct proinflammatory effects on airway epithelial cells. These proinflammatory effects are abolished by serine inhibitors [26], suggesting the involvement of a serine protease. However, the presence and activities of proteases in cockroach extracts, especially those targeting aspartate, cysteine, and serine, remain controversial [27].

2.4. Fungi. A large number of mold species are known to harbor proteases. Serine proteases of airborne fungi have been identified in *Penicillium*, *Aspergillus*, *Rhodotorula*, *Curvularia*, and *Cladosporium* species [28–31]. Cross-reactivity has been reported among fungal species [32–35]. The active protease of *Epicoccum purpurascens*, Epi p 1, which is a potent fungal allergen source inducing respiratory allergic disorders worldwide, also plays an important role in driving allergic responses in the airways of murine models [36].

Recent research has focused on the role of exogenous allergen proteases in allergic disorders. Enzymatic activities have been proposed to facilitate sensitization to various allergens [37–39].

3. Disruption of Epithelial-Cell Barrier

In the clinical setting of asthma, there is evidence that the barrier function of the airway epithelium is impaired [40–43]. The airway epithelium serves as a barrier via the formation of tight junctions (TJs) which seal off the paracellular space. TJs also have gate functions that regulate the passage of ions and macromolecules through the paracellular pathway. TJs are comprised of a series of interacting proteins and receptors including zonula occludens (ZOs) proteins ZO-1–3, occludin, claudins 1–5, and transmembrane adhesion proteins (β-catenin, E-cadherin, and junctional adhesion molecule-1). These proteins and receptors appear to interact in a homophilic manner. ZO-1, -2, and -3 bind to the cytoplasmic tail of occludin and link the TJ to the actin cytoskeleton. Occludin appears to copolymerize to form claudin-based TJ strands. Claudins adhere to each other in a homotypic as well as a heterotypic manner, determining the barrier properties of cell-cell contact existing between two neighboring cells, and regulate paracellular permeability. Regulatory molecules, including tyrosine kinases, proteases, and GTPases, colocalize near the tight junction. Coordinated functions between the transmembrane components and cytoplasmic molecules, along with the cytoskeleton and regulatory molecules, play a crucial role in not only barrier function but also communication between adjacent cells as well as in the regulation of intercellular transport [44, 45].

Initiation of sensitization to allergens in the airway is preceded by their uptake and processing by a subpopulation of mucosal dendritic cells (DCs), followed by presentation of specific peptide epitopes to naïve T cells in association with major histocompatibility (MHC) class II. Mucosal DCs are positioned within the epithelium. DCs extend their processes between epithelial cells directly into the airway lumen, as a periscope function that allows continuous immune surveillance of the airway luminal surface. DCs form TJs with epithelial cells through their expression of adhesion molecules and via E-cadherin homotypic interactions [46, 47].

DCs act as immune sentinels by alerting T cells to the presence of antigens after delivery and presentation to draining lymph nodes. In mice, antigen administered into the lungs is rapidly, that is, in as little as 12 hours, transported to thoracic lymph nodes [48, 49]. The path taken by inhaled antigens from the airways to sampling by DC subsets has yet to be characterized in detail. Antigen sampling functions may also differ between DCs located in the alveolar wall and mucosal DCs that line the conducting airways [47, 50–52].

Although the sampling function of airway DCs ensures that any inhaled protein will be recognized and presented to T cells, allergen source-derived proteases compromise epithelial barrier function by degrading TJ proteins, thus facilitating allergen delivery across epithelial layers.

Proteases released by major allergenic pollens have been shown to injure airway epithelial cells *in vitro* [53]. Proteolytic enzymes contained in pollens of giant ragweed (*Ambrosia trifida*), white birch, Kentucky blue grass, and Easter lily (*Lilium longiflorum*) facilitate allergen delivery across epithelia by degrading occludin, resulting in disruption of epithelial TJs. This effect was blocked by inhibitors of serine and cysteine proteases in Madin-Darby canine kidney (MDCK) and Calu-3 cells [54].

Der p 1 increased epithelial permeability by disrupting TJs [55]. Immunoblotting demonstrated that the disruption of TJ morphology by Der p 1 was associated with cleavage of ZO-1 and occludin in MDCK and 16HBE14o-human bronchial epithelial cell lines [56]. Putative Der p 1 cleavage sites were found in peptides from an extracellular domain of occludin and in the TJ adhesion protein claudin-1. Extracellular cleavage of TJs initiates intracellular processing of junctional constituents. Der p 1 is also envisaged to

operate indirectly on TJs by activating a cell surface zymogen, which then proceeds to cleave TJs [57]. ZO-1 is intracellular and is therefore unlikely to be directly degraded by Der p 1, and its breakdown is presumed to be a consequence of TJ disassembly [57].

Der p 1 and *Dermatophagoides farinae* 1 (Der f 1) can inactivate lung surfactant proteins (SP)-A and -D [58], which are predominantly synthesized and secreted in the lung by alveolar type II cells and Clara cells. SP-A and-D are known to play not only significant roles in innate immune defense such as bacterial aggregation and modulation of leukocyte function, but also are implicated in the allergic response [59, 60].

Allergens, derived from cockroach extracts, are reported to increase the permeability of bronchial airway epithelial cells indirectly through the induction of vascular endothelial growth factor [61] and thereby gain access to intraepithelial DCs.

Aspergillus fumigatus proteinase directly induces human epithelial cell detachment [62]. Pen ch 13, a major allergen of *Penicillium chrysogenum,* is a serine protease. Its enzymatic activity damages the epithelial barrier by cleaving the TJ protein occludin at Gln202 and Gln211, amino acids within the second extracellular domain of the protein on 16HBE14o-cells [63], followed by the induction of proinflammatory responses in epithelial cells.

Epithelial injury and aberrant repair are involved in triggering asthma. Interestingly, Pen ch 13 decreases cell surface expression of CD44 in 16HBE14o-cells and primary bronchial epithelial cells [64], which has been suggested to contribute to repair of epithelial damage [65]. CD44 is a transmembrane adhesion molecule and the major receptor for hyaluronan, a major extracellular matrix component. CD44 is important for the removal of extracellular matrix from sites of tissue injury, and impaired clearance of hyaluronan results in persistent inflammation [66, 67].

Thus, loss of epithelial barrier function, as a consequence of proteases associated with allergens, facilitates antigen access to DCs. The result is that the adaptive immune response is skewed towards Th2 cells, and the IgE immune response is amplified.

Most results are based on in vitro study. Since the digestion process of proteases needs a suffientcient local concentration and time, dilution in mucus, as well mucociliary clearance of the respiratory tract, may complicate the digestion process of TJs in vivo.

4. Allergen Source-Derived Proteases Activate Pattern Recognition Receptors (PRRs)

Pulmonary epithelial cells are now thought to play vital roles in both innate and adaptive immune responses. Epithelial cells can sense and respond to inhaled allergens or proteases via activation of a variety of pattern recognition receptors (PRRs) such as TLR and PAR. These activated receptor signals trigger nuclear factor κB (NF-κB) activation, leading to transcriptional activation of several proinflammatory genes including those encoding cytokines and chemokines. Epithelial production of thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-33 and IL-25, as well as the production of chemokines, both attract and activate DCs, skewing T-cell production toward to the Th2 subset.

5. PAR-2

Protease allergens are reported to elicit non-IgE-mediated airway reactions by triggering innate immunity receptors, such as PARs, to activate epithelial cells, mast cells, and DCs, which in turn leads to further release of mediators [68]. PARs constitute a novel family of seven-transmembrane G-protein-coupled receptors. To date, four PARs have been identified and cloned. They are widely expressed on cells comprising blood vessels, connective tissue, epithelium, and airways, as well as on leukocytes [69]. PARs are activated by proteolytic cleavage at the amino terminus, allowing interaction between the newly formed "tethered ligand" and the second extracellular loop of the receptor. This interaction confers a cellular signaling property. PARs can also be activated by small peptides that mimic the tethered ligand. Activated PARs coupled to G-signaling cascades increase phospholipase C level, which in turn raises intracellular calcium (Ca^{2+}) level [70–72]. G protein activation also generates a transcriptional response through extracellular signal-regulated and mitogen-activated protein kinases, as well as NF-κB [73–75].

In patients with bronchial asthma, PAR-2 expression is increased on the surface of respiratory epithelial cells [76, 77]. PAR2 agonists induce constriction of human bronchi [78]. Lack of PAR-2 expression is reported to lower inflammatory cell infiltration and reduce airway hyperreactivity in response to allergen challenge in mice [79].

Asthma is associated with increased water and chloride (Cl^-) secretion into the airway lumen due to elevated expression of Ca^{2+}-activated Cl^- channels [80–82]. Stimulation of PAR-2 receptors in mouse and human airways inhibited amiloride-sensitive sodium (Na^+) conductance and stimulated luminal Cl^- channels and basolateral potassium (K^+) channels, which together may cause accumulation of airway surface fluid [83].

Activation of PAR-2 was, however, shown to reduce airway inflammation in a rabbit model of experimental asthma [84], which supports the concept of PAR-2 being a cytoprotective receptor involved in prostanoid-dependent cytoprotection in the airways. Prostaglandin E (PGE), which inhibits pulmonary infiltration by immune cells and bronchial constriction in allergen-induced asthma, is produced by cultured airway smooth muscle cells [85, 86], as well as airway epithelial cells, follicular DCs, fibroblasts, monocytes, and alveolar macrophages [87]. Intranasal administration of PAR-2-AP was shown to inhibit airway eosinophilia and hyperresponsiveness in allergic mice via cyclooxygenase- (COX-) 2-dependent generation of PGE_2 [88].

Thus, whether the activation of PAR-2 promotes or opposes the progression of airway inflammatory responses depends on the experimental model and species and is not yet fully understood. This is an area requiring further research.

PAR-2 is a major candidate for sensing environmental exposure to serine proteases. PAR-2 is involved in antigen-induced asthmatic responses, including increase in IgE production, a heightened methacholine response, upregulated production of IL-6, IL-8, GM-CSF, and eotaxin, increased matrix metalloproteinase-9 (MMP-9) release, and relaxation of bronchi [89, 90]. Interestingly, MMP-9, which plays an important role in remodeling of the airways in disease, is hypothesized to exert its effects on the epithelium by cleaving one or more components of cell-cell junctions and triggering anoikis [91].

Increased release of proinflammatory cytokines, such as IL-6 and IL-8, from airway epithelial cells in response to proteases contained HDM [92, 93]. Der p 3 and Der p 9 may induce a non-allergic inflammatory response in the airways, via release of proinflammatory cytokines from the bronchial epithelium, which is at least partially mediated by PAR-2 [94]. Although release of IL-6 and IL-8, due to the protease activity of Der p 1, can occur via a mechanism independent of Ca^{2+} mobilization and PAR activation [95, 96], their release from an A549 cell line was reported to be associated with PAR-2 [97].

Fungal proteases, from *Aspergillus fumigates*, *Alternaria alternate,* and *Cladosporium herbarum*, differentially induced morphologic changes, cell desquamation, and the production of various cytokines [98, 99]. The protease activity of Pen ch 13, an allergen from *Penicillium chrysogenum*, is required for the induction of PGE2, IL-8, transforming-growth-factor- (TGF-) beta1 and COX-2 expression in A549 cells, 16HBE14o-cells, and primary cultures of HBEpC [63]. Pen c 13, the main allergen produced by *Penicillium citrinum*, induces the expression of IL-8 in human airway epithelial cells by activating either PAR-1 or PAR-2 [100].

Fecal remnants [101] and extracts [102] of German cockroach induced mucosal allergic sensitization and inflammation via PAR-2 in mice. Inflammatory responses of human eosinophils to German and Oriental cockroach (*Blatta orientalis*) extract antigens are mediated via PAR-2 [27, 103]. Recent data provide evidence implicating the protease activity of cockroaches in cytokine regulation. Allergens of German and American cockroaches induce IL-8 expression in H292 cells [104] and A549 cells [105], respectively, and both are blocked by serine protease inhibitors, suggesting PAR-2 might play a role in cockroach allergen-induced IL-8 secretion from human airway epithelial cells [105]. Proteases in German cockroach extract regulate PAR-2 and extracellular signal-regulated kinase (ERK) to increase NF for IL-6 (NF-IL6) activity (recently known as C/EBP-β), as well as synergistically regulating TNF-α-induced IL-8 promoter activity in the human airway epithelium [104, 106–108]. German cockroach fecal remnants contain active serine proteases, which augment TNF-α-induced MMP-9 expression via a mechanism that involves PAR-2, ERK, and AP-1 [109].

Interestingly, exposure to inhaled antigens with a PAR-2-activating peptide led to allergic sensitization, whereas exposure to Ag alone induced tolerance in BALB/c mice administered ovalbumin (OVA), suggesting PAR-2 activation in the airways at the time of inhaled antigen exposure to be capable of shifting the resulting immune response toward allergic sensitization and the development of asthma. Furthermore, PAR-2-mediated allergic sensitization is reported to be TNF dependent [110].

6. Thymic Stromal Lymphopoietin

In addition to serving as a physical barrier, airway epithelial cells are now thought to play essential roles in allergic responses. TSLP [111] is expressed mainly by epithelial cells comprising the barrier surfaces of the lungs. Genetic analyses of atopic populations have demonstrated polymorphisms in TSLP to be associated with asthma and airway hyperresponsiveness, IgE concentration, and eosinophilia [112–116]. Overexpression of TSLP in the lungs can trigger Th2 cell immunity. Mice expressing TSLP in the lungs spontaneously develop an airway inflammatory disorder with characteristics similar to those of human asthma [117].

Studies of endobronchial biopsy specimens and BAL fluid of subjects with severe asthma have shown that asthma is associated with elevated bronchial mucosal expression of TSLP and Th1-attracting (IP-10/CXCL10) and Th2-attracting (TARC/CCL17, MDC/CCL22) chemokines [118, 119].

IL-25 and TSLP perform important functions in the initiation of allergic responses [120–122]. TSLP expression is induced in airway epithelial cells by exposure to allergen-derived proteases, and PAR-2 is involved in this process. A recent study demonstrated upregulation of IL-25 and TSLP mRNA in pulmonary epithelial cells after protease allergen treatment *in vivo* and *in vitro*, and that the induction of IL-25 and TSLP occurs via the intracellular ERK and p38 MAP kinase pathways [123]. TSLP induces the innate immune functions of DCs, leading to chemokine-driven recruitment of Th2 cells to the airway, and these cells then produce Th2 type cytokines. TSLP also triggers the maturation of DCs and their migration to mediastinal lymph nodes, again skewing the T-cell distribution in favor of inflammatory Th2 cells producing IL-4, IL-5, IL-13, and TNF-α. These processes involve interactions between costimulatory molecules, such as OX40 (CD134) in the membranes of naïve T cells and OX40L (CD134L) in the membranes of DCs [124, 125]. TSLP was reported to be induced in the airway epithelial cell line BEAS-2B by exposure to *Alternaria* proteases [126] (see Figure 1).

Basophils are directly activated by protease allergens and produce TSLP. Cysteine protease activity of papain, an occupational allergen homologous to Der f 1 and Der p 1, was reported to initiate Th2 sensitization *in vivo* in mice via activation of basophils [122].

7. Allergen Source-Derived Proteases Enhance Sensitization to Other Allergens and Allergen Components

The tertiary structure of an allergen is involved in IgE-binding activity. The tertiary architecture of the Der p 1 molecule itself is not sufficient to induce major production

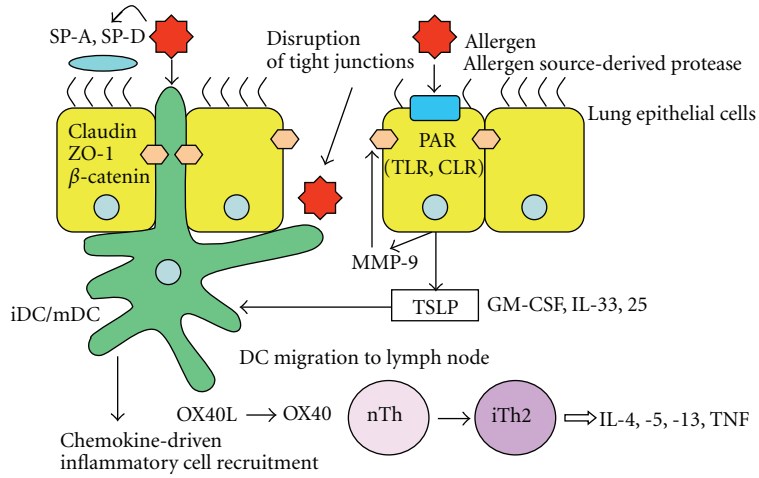

Figure 1: Allergen source-derived proteases compromise epithelial barrier function by degrading TJ proteins, facilitating allergen accessibility to DCs. Enzymatically active allergens can activate PAR to induce TSLP. TSLP induces immediate innate immune functions in DCs, leading to recruitment of inflammatory cells. TSLP triggers the maturation of DCs, so they migrate to mediastinal lymph nodes. Induction of DCs to upregulate OX40L by TSLP promotes Th2 responses. PAR also upregulates production of MMP-9, which degrades tight junction proteins. Thus, impairment of airway epithelial barrier function and activation of epithelial cells are involved in the pathogenesis of inflammation mediated by allergen source-derived proteases.

of both IgE and IgG, but its proteolytic activity is crucial for eliciting positive immune responses in naïve mice [127].

Immunization of mice with proteolytically active Der p 1 results in significant increases in total IgE and Der p 1-specific IgE synthesis, as compared with animals immunized with Der p 1 irreversibly blocked with E-64, a cysteine protease inhibitor [128]. The proteolytic activity of Der p 1 heightens inflammatory cell infiltration into the lungs and systemic IgE production when administered directly into the respiratory tract [129].

Allergens with protease activity are also able to mediate sensitization to nonprotease proteins. Exposure to HDM extract establishes a mucosal environment fostering the development of allergic sensitization to otherwise weak or innocuous antigens, such as OVA, suggesting that whether an airborne allergen will generate allergic airway disease may depend, at least in part, not simply on being exposed to it but rather the setting in which that exposure takes place [130, 131].

Active protease contents of fungal extracts can influence the induction and severity of allergic airway disease in mice. Proteolytically active molecules can facilitate the presentation of nonproteolytic allergens to the immune system, thereby augmenting sensitization to allergens. These proteolytic allergens thereby promote Th2 cell sensitization. For a mature response, the participation of components such as enzymes may have a major role, as is suggested by the response reported with crude antigen and recombinant allergens. Alkaline serine proteases are major allergens of *Aspergillus* species. The alkaline serine protease allergen of *A. fumigatus* (Asp f 13) induces IgE as well as an inflammatory response and has synergistic effects on the Asp f 2-induced immune response in mice [31, 132].

8. Allergen Source-Derived Proteases Cleave Cell Surface Molecules:Roles beyond the Airways

Allergen source-derived proteases have been recognized as having the ability to cleave key regulatory molecules in allergic reactions involving cell surfaces, and to amplify IgE responses.

Previously suggested pathogenic roles of exogenous proteases, especially Der p 1, involve cleavage of various endogenous proteins, including removal of low-affinity IgER (CD23) from the surface of human B lymphocytes. This loss of cell surface CD23 from IgE-secreting B cells may promote and amplify IgE immune responses by eliminating an important inhibitory feedback mechanism that would normally limit IgE synthesis. Furthermore, fragments of CD23 released by Der p 1 may directly promote IgE synthesis [133].

Der p 1 cleaves the α subunit of the IL-2 receptor (IL-2R or CD25), which is pivotal for Th1 cell propagation, removing it from the surface of human peripheral blood T cells. As a result, these cells show markedly diminished proliferation and interferon γ secretion in response to a potent stimulus such as anti-CD3 antibody. IL-2R cleavage by Der p 1 is likely to cause impaired growth of cells of the Th1 subset and may, as a consequence, bias the immune response toward Th2 cells [134, 135].

Der p 1 also cleaves cell surface DC-SIGN and DC-SIGNR, which are closely related C-type lectin transmembrane receptors expressed within compartments of the immune system. These molecules then bind to intracellular adhesion molecule-2 (ICAM-2), expressed on endothelial and T cells, and ICAM-3, expressed on T cells. Both are

involved in DC trafficking, DC-T-cell interactions, and skewing of the immune response in favor of Th 1 [136].

Proteolytic activity of Der p 1 results in cleavage of CD40 from the DC surface. This deprives DCs of the ability to receive CD40L-mediated signals from T cells, which is an important pathway stimulating IL-12 production. This downregulation of IL-12 may enable DCs to directly promote the differentiation of naïve T cells toward the Th2 cytokine profile [137].

9. Conclusion

Although genetic aspects of airway epithelium barrier deficiency have yet to be determined, both structural and functional abnormalities of the epithelium underlie the pathogenesis of bronchial asthma. Protease activity in allergens confirms that allergenicity not only results from the reaction to an epitope, which is involved in adaptive immune responses by T and B cells, but also from disruption of airway barrier function and activation of innate immune responses through epithelial cells [138–140]. Stimulation of PAR-2 signaling by protease allergens participates in the inflammatory process, and may serve as a link between innate and adaptive immune responses.

Analysis of these allergen proteases, which constitute protease-sensing pathways in airway epithelial cells, is essential for elucidating the pathogenesis of allergic asthma. A full understanding of these processes is anticipated to lead to both treatment and preventive measures against asthma development.

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Research Article

Calreticulin Is a Negative Regulator of Bronchial Smooth Muscle Cell Proliferation

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Background. Calreticulin controls the C/EBPαp42/p30 at the translational level trough a cis-regulatory CNG rich loop in the CEBPA mRNA. We determined the effects of steroids and long-acting beta-agonists on the p42/p30 ratio and on calreticulin expression in primary human bronchial smooth muscle (BSM) cells. *Methods*. The effects of budesonide (10^{-8} M) and formoterol (10^{-8} M) were studied in BSM cells pre-treated with siRNA targeting calreticulin. The expression of C/EBPα and calreticulin was determined by immuno-blotting. Automated cell counts were performed to measure proliferation. *Results*. All tested BSM cell lines ($n = 5$) expressed C/EBPα and calreticulin. In the presence of 5% FBS, the p42/p30 ratio significantly decreased ($n = 3$, $P < 0.05$) and coincided with BSM cell proliferation. High levels of calreticulin were associated with a decreased p42/p30 isoform ratio. FBS induced the expression of calreticulin ($n = 3$, $P < 0.05$), which was further increased by formoterol. siRNA targeting calreticulin increased the p42/p30 ratio in non-stimulated BSM cells and significantly inhibited the proliferation of PDGF-BB-stimulated BSM cells ($n = 5$, $P < 0.05$). Neither budesonide nor formoterol restored the p42 isoform expression. *Conclusions*. Our data show calreticulin is a negative regulator of C/EBPα protein expression in BSM cells. Modulation of calreticulin levels may provide a novel target to reduce BSM remodeling.

1. Introduction

An important feature of asthma pathology is airway wall remodeling, characterized by a thickened basement membrane and an increase with respect to the bulk of the bronchial smooth muscle (BSM) cells [1–3]. Earlier we showed that the increased proliferation rate of asthmatic BSM cells was normalized after the introduction of an expression vector for full length C/EBPα mRNA [4]. We have further provided data showing a disease specific expression of C/EBP-isoforms in asthma and COPD [5].

C/EBPα can be expressed as full length and truncated protein isoforms, commonly referred to as p42 and p30. The full length C/EBPα (p42) functions as a proliferation inhibitor, whereas the truncated C/EBPα (p30) does not have this effect [6, 7]. A decreased p42/p30 ratio may therefore render BSM cells a growth advantage and result in thicker layers of muscles around the airways as observed in the lungs of asthma patients.

The standard therapy for asthma consists of drugs that reduce airway inflammation (predominantly glucocorticoids) and induce relaxation of the smooth muscles (predominantly β_2-agonists). We have earlier shown the molecular biological basis of the interaction of both classes of drugs, which involved the formation of a complex consisting of the glucocorticoid receptor and C/EBPα [8–11]. This complex is able to activate the cell cycle inhibitor $p21^{cip1/waf1}$ [9, 10], thus demonstrating an interactive negative regulatory network for cell proliferation. The observed diminished expression of C/EBPα in BSM cells of asthma patients is mainly due to posttranscriptional regulation affecting the translation of the *CEBPA* mRNA [12, 13].

In general, two mechanisms can be involved in translation control: "global" and "selective". Global control acts on all mRNAs in a nonspecific manner, whereas selective translation regulation targets a specific subset of mRNAs. These specific mRNAs often have *cis*-regulatory sequences that sense subtle changes in the activity of the translation

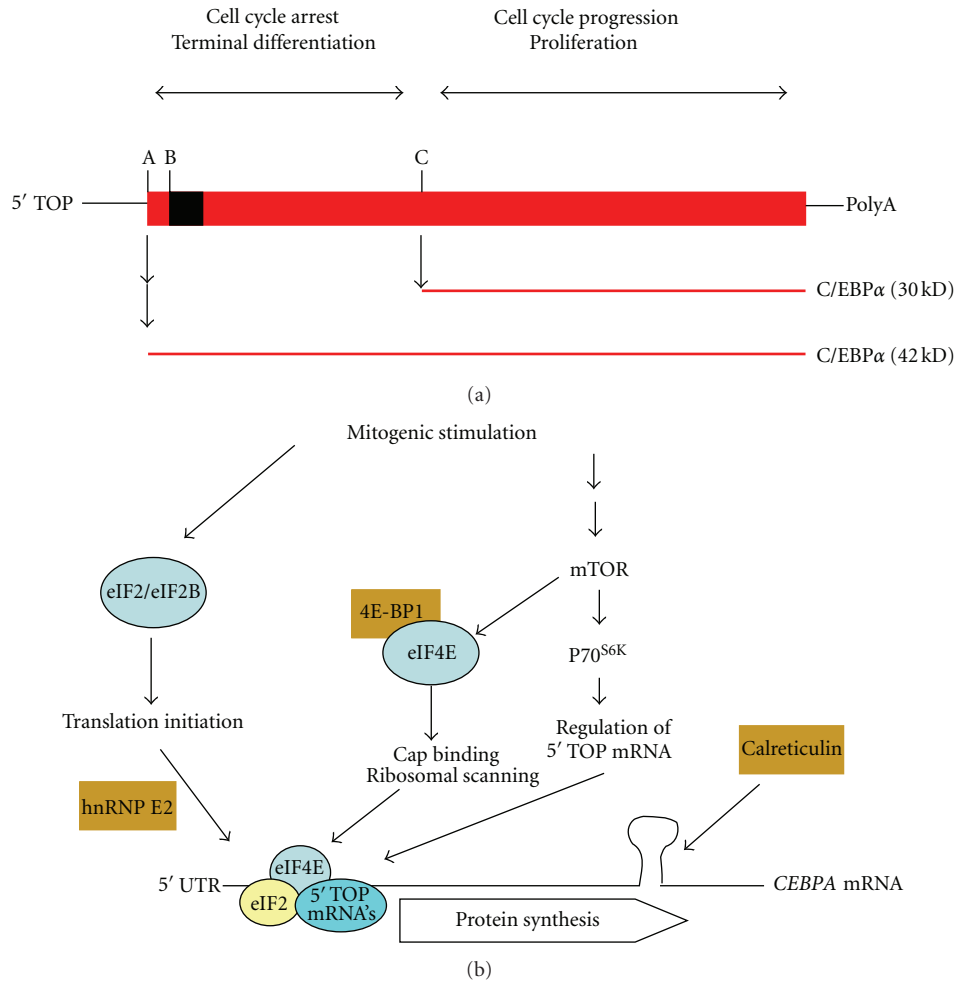

Figure 1: (a) Simplified scheme of the *CEBPA* mRNA. Due to alternative translation start sites (A, B, and C), full length (p42) and truncated (p30) C/EBPα proteins with distinct functions can be formed. Start site B is out of frame and determines whether A or B is accessible for translation, hence producing either p42 or p30 C/EBPα proteins. (b) Schematic representation showing three important signaling pathways for the translation control of *CEBPA* messenger RNA: (1) the pathway leading to activation of the eukaryotic initiation factors eIF2 and eIF2B, which is counteracted by hnRNPE2, (2) the pathway of mTOR and eukaryotic initiation factor 4E (eIF4E), which is inhibited by 4E-BP1, and (3) the pathway leading to calreticulin (CRT) expression, a protein that binds to a double-stranded RNA loop and prevents the translation of full length C/EBPα proteins. Abbreviations: 5′ TOP: 5′ tract of pyrimidine; mTOR: mammalian target of rapamycin.

machinery or form loops that affect the accessibility of the appropriate translation start sites. It is now well documented that the *CEBPA* mRNA can be expressed as a full length protein (p42) or a truncated form (p30) [6, 7, 14–18]. The p42/p30 ratio is predominantly controlled at the translational level [15–18]. Full lengths (p42) and truncated (p30) C/EBPα proteins are generated from one single 5′ tract of pyrimidine (5′ TOP) *CEBPA* mRNA (Figure 1(a)). Three important signaling pathways regulate the translation of 5′ TOP messengers (Figure 1(b)). The first is the ubiquitous eukaryotic initiation factor 2 (eIF2). The second is leading to activation of mammalian target of rapamycin (mTOR) and subsequent activation of eukaryotic initiation factor 4E (eIF4E). These pathways are stringently controlled by specific inhibitory proteins, including hnRNPE2, which interferes with translation initiation, and 4E-BP1, a protein that prevents ribosomal scanning [19]. The third level of control of *CEBPA* mRNA translation is found in a *cis*-regulatory double-stranded RNA loop, which provides a docking site for calreticulin. When calreticulin is bound to this sequence, translation of the full length C/EBPα (p42) is reduced [20].

Here, we isolated and maintained primary human BSM cells and studied the involvement of calreticulin in the regulation of *CEBPA* mRNA translation and whether budesonide and formoterol are able to modulate the p42/p30 ratio.

2. Material and Methods

2.1. Tissue Specimens and Cell Cultures. Lung tissue specimens were obtained from the Department of Internal Medicine, Pneumology, and the Department of Thorax Surgery, University Hospital Basel, Switzerland, with the approval of the local Ethical Committee and written consent of all patients. BSM cells were established as previously described

[10] and grown in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS), 8 mM L-glutamine, 20 mM HEPES, and 1% MEM vitamin mix (Gibco, Paisley, UK). Neither antibiotics nor antimycotics were added at any time.

2.2. Cell Treatment and Drugs. Confluent BSM cells were cultured for 24 hours in the presence or absence of 5% FBS and grown in the presence of an optimal concentration [4, 8–10] of budesonide (10^{-8} M), formoterol (10^{-8} M), or a combination of both drugs for 24 and 96 hours.

2.3. Small Inhibitory RNA (siRNA) Treatment. Transfection with siRNA for calreticulin or negative control (Ambion, Austin, USA) was performed according manufacturer protocol. Cells (70% confluence) were plated into 6 well plates and transiently transfected with siRNA (50 nM) for 6 hours. Thereafter, fresh RPMI was added for 24 hours. Then, cells were cultured in presence or absence of budesonide (10^{-8} M) or formoterol (10^{-8} M). Cell lysates were collected after 24 hours and prepared for immuneblot analysis.

2.4. Protein Isolation and Analysis by Immunoblot. Cellular proteins were isolated from confluent cells by dissociation in lysis buffer (62,5 mM Tris-HCl (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 10% glycerol) and denaturation in sample buffer (3x Laemmli buffer with β-mercapto-ethanol) and boiling for 5 min. Equal protein amounts were loaded onto a 4–12% PAGE-gel (Pierce Biotech, Thermo Fisher Scientific, Rockford, IL, USA) and were size fractionated by electrophoresis (1 hr, 100V, open A). The gel was sandwiched between two nitrocellulose membranes (Biorad, Reinach, Switzerland), and proteins were transferred (transfer buffer: 0.05 M NaCl, 2 mM Na-EDTA, 0.1 mM DTT, 10 mM, Tris HCl (pH 7.5)) overnight (50°C). Protein transfer and equal loading were confirmed by Ponceau's staining. The membranes were blocked (10 min) in 3% bovine serum albumin (Roche, Rotkreuz, Switzerland) in 1x phosphate buffered saline with 0.05% Tween-20 (PBST). The membranes were incubated (1 hour) at room temperature (RT) with one of the antibodies to C/EBPα (Santa Cruz Biotech, Santa Cruz, USA) and calreticulin (Santa Cruz Biotech). Membranes were then washed (3 × 5 min) and incubated (1 hour, RT) with horseradish labeled species-specific antibodies (Santa Cruz Biotech). The membranes were washed (3 × 5 min) with and incubated (5 min) with ECL-substrate (Pierce), and protein bands were visualized on X-ray films (Fuji Film, Medical X-ray film, Luzern, Switzerland). Protein bands were semiquantified by an image analysis system (ImageJ). Protein expression was normalized to α-tubulin as internal control. The presented p42/p30 ratios were calculated from normalized densitometry data.

2.5. Proliferation. BSM cells were plated in a 24 wells plate at a density of 10^4 cells/well. Next cells were grown for 24 hours in the presence of FBS (5%), before being serum starved for 24 hours. Then, cells were incubated in absence or presence of FBS (5%) for 96 hours. After trypsinization cells, were counted manually and/or by using an automated particle counter (Coulter).

2.6. Statistics. Cytokine and proliferation data are presented as mean ± SEM, immunoblot analysis is shown as mean ± SEM after densitometric image analysis (ImageJ software, National Institute of Mental Health, Bethesda, MD, USA) of independent experiments. Paired/unpaired Student's t-test was performed, and P values <0.05 were considered significant.

3. Results

3.1. Effects of FBS, Budesonide, and Formoterol on p42/30 Ratios. To determine the effect of FBS, budesonide, and formoterol on p42/p30, BSM cells ($n = 3$) were cultured for 0, 24, and 96 hours in growth medium (5% FBS) supplemented with either budesonide (10^{-8} M) or formoterol (10^{-8} M). FBS (5%) significantly reduced p42 C/EBPα levels ($n = 3$; $P < 0.05$), both after 24 and 96 hours (Figure 2(a)). Concomitantly, p30 C/EBPα levels were significantly increased ($n = 3$; $P < 0.05$) at both time points, resulting in reduced p42/p30 ratios which are presented in Figure 2(b). The addition of formoterol to FBS stimulated BSM cells further reduced the p42/p30 ratio below 0.01, whereas budesonide did not modify the effect of FBS (Figure 2(b)). As shown in Figure 3(c), a reduced p42/p30 ratio coincided with a significantly increased proliferation rate of 5% FBS-stimulated BSM cells relative to nonstimulated cells ($n = 4$; $P < 0.05$).

3.2. Calreticulin Levels Coincide with C/EBP p42/p30 Ratios. BSM cells were incubated with FBS (5%) supplemented with budesonide (10^{-8} M) or formoterol (10^{-8} M). As demonstrated in Figure 3(a), resting BSM cells expressed low levels of calreticulin, which were significantly upregulated after 24 an 96 hours in the presence of 5% FBS, only. After 96 hours, budesonide (10^{-8} M) slightly restored the expression of the p42 isoform, but the p42/p30 ratio was unaffected. The expression of calreticulin protein coincided with low levels of C/EBPα (p42) and high levels of C/EBPα (p30): the p42/p30 ratio significantly decreased ($n = 3$; $P < 0.05$). As shown in Figure 3(b), calreticulin-specific siRNA significantly decreased the expression of calreticulin protein ($n = 3$, $P < 0.05$). The knockdown of calreticulin only increased the expression of C/EBPα (p42) in untreated BSM cells. Finally, BSM cells were transfected with increasing concentrations of calreticulin-specific siRNA and incubated in presence and absence of PDGF-BB for 96 hours. As demonstrated in Figure 3(c), siRNA for calreticulin dose-dependently inhibited the proliferation of PDGF-BB-stimulated BSM cells ($n = 5$; $P < 0.05$ for calreticulin-siRNA ranging from 0.1 to 5.0 ng/mL), whereas the control siRNA did not have an effect on PDGF-BB-induced proliferation ($n = 5$; $P = 0.59$).

4. Discussion

An increased capacity to proliferate is a key feature of BSM cells obtained from asthma patients and may provide an

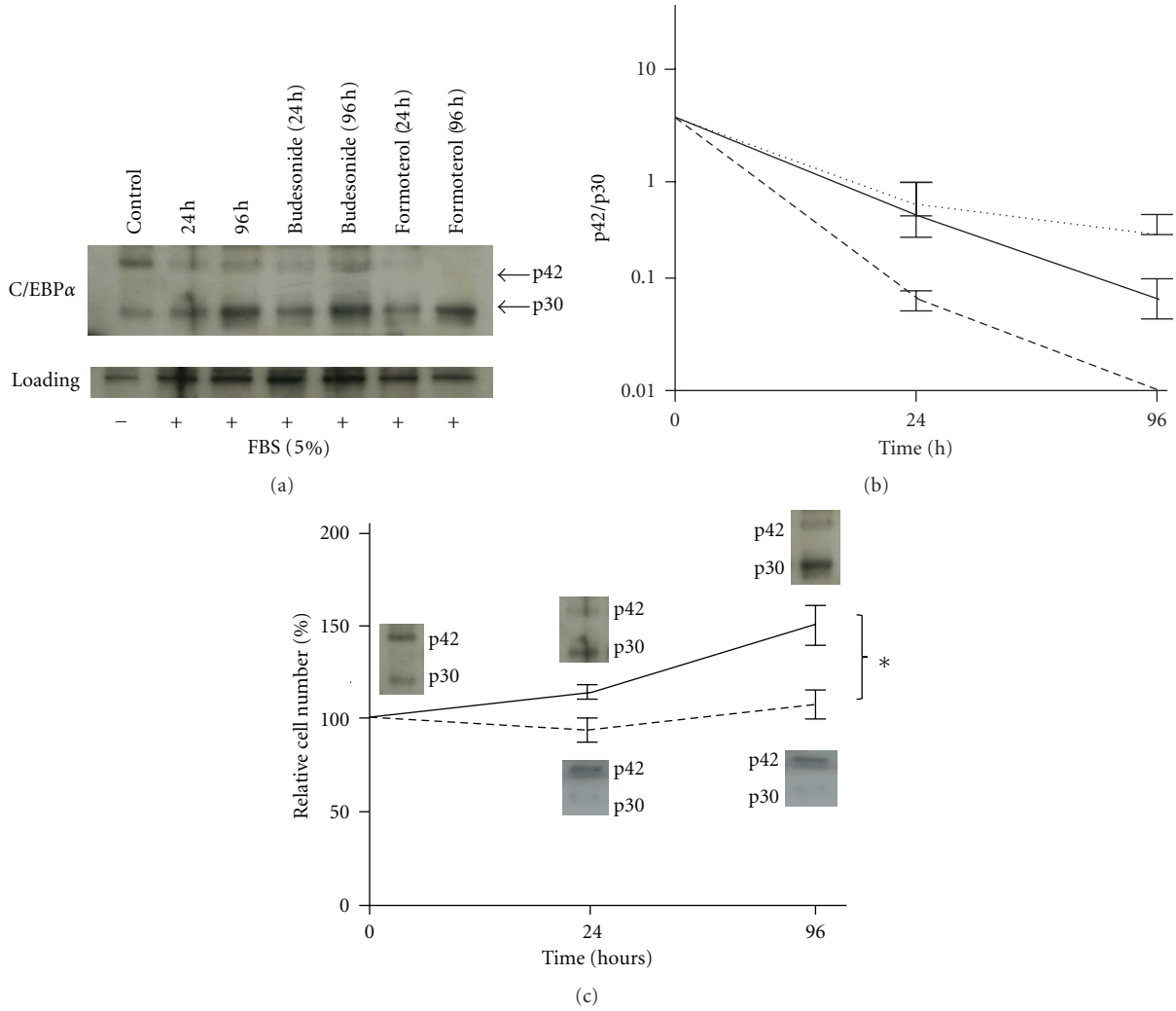

Figure 2: (a) Representative immunoblot analysis demonstrating the modulatory effect of asthma drugs on the C/EBPα (p42) and C/EBPα (p30) expression pattern in human BSM cells. BSM cells were untreated (control) or incubated with 5% FBS alone, and in the presence of budesonide (10^{-8} M) or formoterol (10^{-8} M) for 24 and 96 hours. Similar data were obtained in two additional cell lines. (b) C/EBPα p42/p30 ratios (mean ± SEM; $n = 3$) in BSM cells cultured for 0, 24, and 96 hours with 5% FBS (solid line), budesonide (dotted line), or formoterol (dashed line). (c) C/EBPα (p42 and p30) expression patterns projected in the proliferation curve of BSM cells. Cells were incubated for 0, 24, and 96 hours in absence (dashed line) or presence of 5% FBS (solid line). Data are expressed as a percentage of the cell number at $t = 0$ (control). *Significant difference between untreated and FBS-stimulated cells ($P < 0.05$; $n = 4$). Photo insets show the corresponding ratio of C/EBPα p42 and p30 expression of one representative experiment at $t = 0$ h (control), $t = 24$ h, and $t = 96$ h.

explanation for the observed increase of BSM bundles surrounding the bronchi of asthma patients [21]. We have extensively explored the role of the *CEBP* transcription factor-family in BSM cell proliferation and concluded that the expression and regulation of C/EBPα isoforms may be crucial to understand the proliferation control of BSM cells [4, 5, 12, 13]. In our present study, we demonstrated that normal BSM cells express C/EBPα (p42) and C/EBPα (p30) isoforms, as well as their specific translation regulator calreticulin. Moreover, we observed a specific relation between these proteins, that is, when calreticulin levels are high, the p42/p30 ratio is small. Furthermore, we showed that in the presence of serum, the p42/p30 ratio significantly decreased. Addition of budesonide, but not formoterol, slightly restored the p42 levels. Restoring p42 levels would theoretically restore the responsiveness of BSM cells to budesonide and/or formoterol, since only full length C/EBPα proteins formed a complex with the glucocorticoid receptor to induce the cell cycle inhibitor $p21^{cip1/waf1}$ [9, 11]. Both budesonide and formoterol were unable to significantly increase the p42/p30 ratio, however. The incapability of budesonide and formoterol to induce the expression of C/EBPα (p42) may explain why the airway remodeling observed in asthma patients is resistant to therapy involving steroids and/or β-mimetics [22].

We have earlier reported that an impaired translation-initiation of the *CEBPA* mRNA in BSM cells of asthma patients was associated with the decreased expression of the translation regulator eIF4E [12]. We were unable to detect significant differences with respect to eIF4E levels between

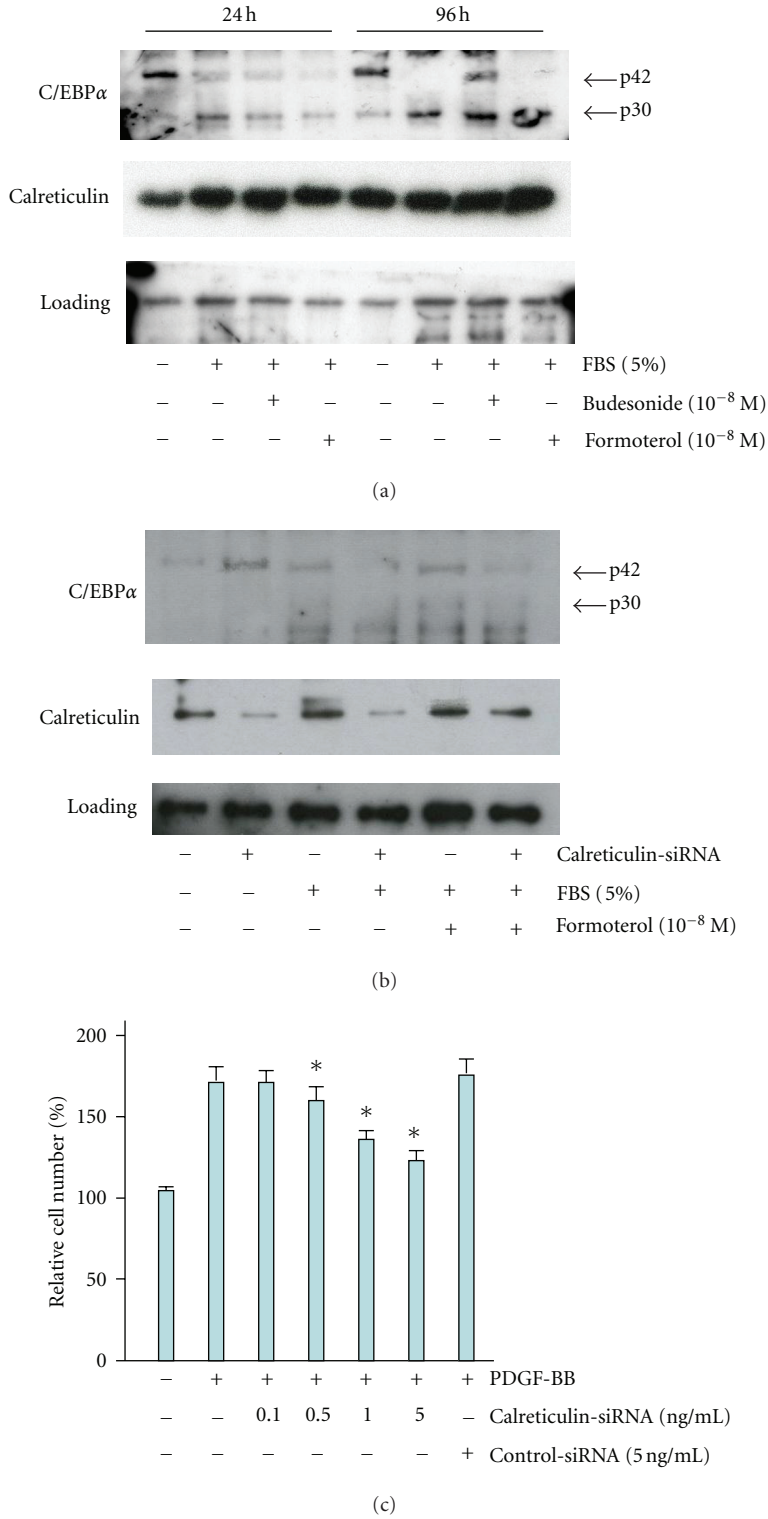

Figure 3: (a) Immunoanalyses demonstrating the effect of asthma drugs on the C/EBPα (p42), C/EBPα (p30), and calreticulin expression pattern. BSM cells were untreated or incubated with 5% FBS alone, and in the presence of budesonide (10^{-8} M) or formoterol (10^{-8} M) for 24 and 96 hours. (b) Immunoanalyses demonstrating C/EBPα (p42), C/EBPα (p30), and calreticulin expression patterns in BSM cells after transient knockout of the calreticulin by siRNA. BSM cells were untreated or incubated with 5% FBS alone, and in the presence of formoterol (10^{-8} M) for 24 hours. (c) BSM cell proliferation (presented as relative cell counts) in response to PDGF-BB (5 ng/mL) and the effect of increasing concentrations calreticulin-specific siRNA relative to control siRNA (as indicated). *Significant inhibition relative to PDGF-BB-stimulated cells ($P < 0.05$; $n = 5$).

house-dust-mite-challenged BSM cells isolated from asthmatic and nonasthmatic subjects, however [13]. Therefore, we proposed that calreticulin, a protein initially identified as an endoplasmic reticulum luminal chaperone that controls the regulation of intracellular Ca^{2+} homeostasis [23], could be pivotal in the downregulation of C/EBPα translation and may be one of the key regulators to explain low levels of C/EBPα proteins in BSM cells of asthma patients. Binding of calreticulin has been shown to inhibit the translation of the *CEBPA* mRNA, as a result of a direct interaction of calreticulin and the *CEBPA* transcript. As depicted in Figure 1(b), calreticulin binds to a stem loop within the *CEBPA* mRNA, which is formed by internal base-pairing of the GCN repeat motif [24]. When calreticulin is bound to this loop, translation of the full length C/EBPα (p42) can no longer be generated and $p21^{cip1/waf1}$ cannot be formed. An inverse relationship of C/EBPα and calreticulin had been demonstrated in adipocytes, where calreticulin inhibited adipogenesis by suppressing the expression of C/EBPα [25]; an observation that was also reported in acute myeloid leukemia [26]. Here, we demonstrated that the same mechanism operates in normal BSM cells, because a transient suppression of calreticulin by siRNA increased C/EBPα (p42) levels in resting BSM cells. It should be noted, however, that in proliferating cells additional mechanisms operate to control C/EBPα isoforms [4, 12, 13]. Therefore, the decrease of the C/EBPα protein level in BSM cells of asthma patients may only partially be related to increased calreticulin.

Our current data show that in the presence of 5% FBS BSM cells rapidly decreased the p42/p30 ratio. Formoterol was able to even further reduce the p42/p30 values. Here, the p42/p30 value went below 0.01, demonstrating an additive effect relative to FBS alone (Figure 2(b)). This additive effect was not observed with budesonide and indicates that formoterol also activates additional pathways not induced by budesonide and independent of C/EBPα (p42). It should be emphasized that both C/EBPα (p42) and C/EBPα (p30) can bind to the same DNA motifs but that p30 cannot exert the antiproliferative effects of p42. The p42, however, is a direct inhibitor of cell cycle progression.

Finally, we found that the siRNA targeting calreticulin dose-dependently inhibited BSM cells proliferation and restored C/EBPα (p42) in nonstimulated BSM cells only. This shows that, although FBS was able to induce calreticulin, it does not exert its effects through an increased expression of calreticulin. Rather, FBS and PDGF may affect the transcription of the gene directly or redirect the translation machinery to alternative start codons present in the *CEBPA* mRNA as described previously [14–16]. Calreticulin levels were slightly increased after 96 hours of treatment with formoterol. The significance of this observation is currently unclear, but may indicate an additional inhibitory effect on proproliferative members of the *CEBP* gene family [24].

Taken together, our current data demonstrate that the translation-controlled C/EBPα (p42) and its counterpart (p30) are present in BSM cells. Calreticulin functions as an important control protein for BSM cell proliferation, but largely independent of the transactivating C/EBPα protein isoform. However, modulation of calreticulin levels—either (epi) genetically or by administration of specific drugs—may be a novel tool to target remodeling parameters involving BSM cells, both *in vitro* and *in vivo*.

Acknowledgments

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Research Article

Cyclin D1 in ASM Cells from Asthmatics Is Insensitive to Corticosteroid Inhibition

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Hyperplasia of airway smooth muscle (ASM) is a feature of the remodelled airway in asthmatics. We examined the antiproliferative effectiveness of the corticosteroid dexamethasone on expression of the key regulator of G_1 cell cycle progression—cyclin D1—in ASM cells from nonasthmatics and asthmatics stimulated with the mitogen platelet-derived growth factor BB. While cyclin D1 mRNA and protein expression were repressed in cells from nonasthmatics in contrast, cyclin D1 expression in asthmatics was resistant to inhibition by dexamethasone. This was independent of a repressive effect on glucocorticoid receptor translocation. Our results corroborate evidence demonstrating that corticosteroids inhibit mitogen-induced proliferation only in ASM cells from subjects without asthma and suggest that there are corticosteroid-insensitive proliferative pathways in asthmatics.

1. Introduction

Asthma is a chronic inflammatory condition of the lung associated with structural remodelling of the airway wall. As a consequence of long-term exposure to inflammatory mediators, the airways of asthmatics become remodelled. Airway smooth muscle mass is increased, and neovascularization is evident in the subepithelial mucosa. Airway fibrosis becomes apparent, with thickening of the lamina reticularis and increased interstitial extracellular matrix deposition being typical features of an asthmatic airway [1]. Although numerous cell types contribute to airway remodelling, the increase in airway smooth muscle mass is considered to have the largest impact on airway narrowing in asthma [2–4].

Inhaled corticosteroids are a first-line anti-inflammatory therapy in asthma. However, as many asthmatics manifest persistent airway hyperresponsiveness even after prolonged corticosteroid therapy [5], corticosteroid resistance and insensitivity is known to exist [6]. Although corticosteroids can inhibit some aspects of remodelling [7], we do not yet know whether ASM mass is reduced by corticosteroid treatment *in vivo*. Interestingly, Roth et al. [8] demonstrated that ASM proliferation in cells derived from asthmatics is resistant to inhibition by corticosteroids, suggesting that the proliferative pathways underlying the hyperplastic ASM phenotype in asthmatics are corticosteroid insensitive.

In this study, we continue investigations into corticosteroid insensitivity *in vitro* by examining the effect of dexamethasone on a key regulator of G_1 cell cycle progression, cyclin D1, in ASM cells from asthmatics and nonasthmatics. Cyclin D1 has been the most widely studied cyclin in ASM biology using cells from nonasthmatics [4, 9, 10] and more recently asthmatics [11]. Our study examines cyclin D1 mRNA and protein expression in ASM cells from asthmatics and demonstrates that cyclin D1 upregulation is insensitive to corticosteroid inhibition.

2. Materials and Methods

2.1. Cell Culture. Human ASM cells were obtained from subjects without and with asthma by methods adapted from those previously described [12, 13], in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. A minimum of three different ASM primary cell lines were used for each experiment.

Table 1: Subject demographics.

Subject no.	Gender	Disease	FEV1 (% Pred)	FVC (% Pred)	FEV1 : FVC (%)	Smoker	Height	Weight	Experiments
1	F	Ca							1, 2
2	F	A	64%	70%	76%	Ex-smoker			1, 3
3	F	Ca							1, 2
4	M	A	82%			No			1, 3
5	F	NSCCa				Yes			1, 2
6	F	A	2.04 L	2.5 L		No	158 cm	64 kg	1, 3
7	M	NSSCa	2.12 L	3.35 L		Ex-smoker	173 cm	76 kg	1, 2
8	F	A	1.87 L	2.32 L		No	168 cm	57 kg	1, 3
9	F	Bronchiolitis obliterans							1, 2
10	M	A	3.75 L	5.9 L		No	179 cm	110 kg	1, 3
11	M	Emphysema							1, 2
12	M	A				No			1, 3
13	F	Emphysema				Yes			1, 2
14	M	A	4.77 L	5.32 L		Yes			1, 3
15	F	Emphysema	27% (0.51 L)	67% (1.5 L)			153 cm		1, 2
16	M	Emphysema, α-1 antitrypsin deficiency	15% (0.61 L)	66% (3.42 L)					4
17	M	A	57%	61%	77%	No			4
18	M	COPD	20%	65%	24%	Yes			4
19	M	Ca + A				Ex-smoker			4
20	M	Ca	1.95 L	2.5 L		Ex-smoker	169 cm	79 kg	4
21	M	A							4

Abbreviations used: Ca, carcinoma; A, asthma; NSCCa, nonsmall cell carcinoma; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 second (% predicted); FVC, forced vital capacity (% predicted).

All the subjects' disease states were confirmed by doctor diagnosis, and subject demographics are shown in Table 1.

Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cyclin D1 mRNA Expression. To examine the time course of induction of cyclin D1 mRNA expression by platelet-derived growth factor BB (PDGF-BB) and repression by dexamethasone, growth-arrested ASM cells from $n = 8$ nonasthmatic and $n = 7$ asthmatic subjects were pretreated for 1 h with 100 nM dexamethasone, compared to vehicle. Cells were then stimulated with PDGF-BB (25 ng/mL: Merck, Darmstadt, Germany) for 0, 2, 4, 8, and 24 h, and cyclin D1 mRNA expression was quantified by real-time RT-PCR as previously described [14].

2.3. Cyclin D1 Protein Expression. To examine the time course of cyclin D1 protein upregulation by PDGF-BB and repression by dexamethasone, growth-arrested ASM cells from $n = 7$ non-asthmatic and $n = 7$ asthmatic subjects were pretreated for 1 h with 100 nM dexamethasone, compared to vehicle. Cells were then stimulated with 25 ng/mL PDGF-BB for 0, 2, 4, 8, and 24 h. Cells were lysed, then cyclin D1 was quantified by western blotting using a rabbit polyclonal antibody against cyclin D1 (M-20: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin as the loading control (mouse monoclonal IgG_1, clone DM 1A; Santa Cruz). Primary antibodies were detected with goat anti-mouse or anti-rabbit horse-radish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). Densitometry was performed using Image J [15].

2.4. Translocation. To measure translocation of the glucocorticoid receptor (GR), growth-arrested ASM cells from $n = 3$ non-asthmatic and $n = 3$ asthmatic subjects were treated with vehicle or dexamethasone (100 nM) for 1 h, prior to stimulation with 25 ng/mL PDGF-BB for 1 h. Cytoplasmic and nuclear protein extraction was performed using NE-PER nuclear and cytosolic extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). GR was quantified by western blotting using a rabbit polyclonal antibody against GR (E-20: Santa Cruz Biotechnology) compared to α-tubulin and a rabbit polyclonal antibody to lamin A/C (cell signaling technology) as a loading control for the cytosolic and nuclear fractions, respectively.

2.5. Statistical Analysis. Statistical analysis was performed using either the Student's unpaired t-test, or one-way or

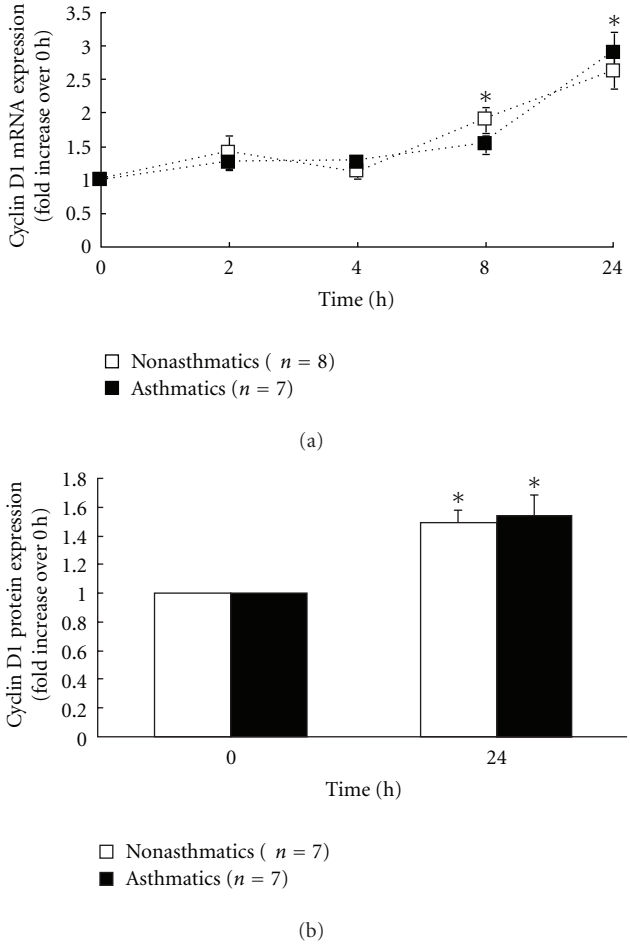

Figure 1: PDGF-BB upregulates cyclin D1 mRNA and protein expression in ASM from nonasthmatics and asthmatics. ASM cells from non-asthmatics and asthmatics were stimulated with 25 ng/mL of mitogen PDGF-BB. (a) demonstrates the temporal kinetics of cyclin D1 mRNA expression quantified by real-time RT-PCR (expressed as fold increase over 0 h). Statistical analysis was performed using one-way ANOVA, followed by Fisher's post hoc multiple comparison test (∗ denotes a significant effect of PDGF-BB on cyclin D1 mRNA, compared to 0 h ($P < 0.05$)). (b) shows densitometric analysis of cyclin D1 protein expression at 24 h quantified by western blotting (expressed as fold increase over 0 h), using α-tubulin as the loading control. Statistical analysis was performed using the Student's unpaired t-test (where ∗ denotes a significant effect of PDGF-BB on cyclin D1 protein, compared to 0 h ($P < 0.05$)). Values are mean + SE.

two-way ANOVA followed by Fisher's post hoc multiple comparison test. P values < 0.05 were sufficient to reject the null hypothesis for all analyses.

3. Results

To examine the time course of induction of cyclin D1 mRNA by the mitogen PDGF-BB, growth-arrested ASM cells from non-asthmatic and asthmatic subjects were stimulated with PDGF-BB for up to 24 h. As shown in Figure 1(a), a significant increase in cyclin D1 mRNA expression was first detected 8 h after PDGF-BB treatment. By 24 h, cyclin D1 mRNA expression had further significantly increased to 2.6±0.3-fold in ASM cells from non-asthmatics and 2.9±0.3-fold in cells from asthmatics ($P < 0.05$). Interestingly, there was no significant difference between the increases in cyclin D1 upregulation in cells from asthmatics, as compared to nonasthmatic controls. Cyclin D1 protein expression at 24 h was similarly upregulated in support of the mRNA data Figure 1(b). Interestingly, there were no significant differences between the amount of cyclin D1 mRNA and protein expression in the asthmatics, as compared to non-asthmatics.

We then examined the effect of the corticosteroid dexamethasone on the temporal kinetics of PDGF-BB-induced cyclin D1 mRNA and protein expression in ASM cells from asthmatics and non-asthmatics. Growth-arrested ASM cells were pretreated for 1 h with vehicle or 100 nM dexamethasone and stimulated with PDGF-BB for up to 24 h. In ASM cells from non-asthmatics, the corticosteroid dexamethasone significantly inhibited cyclin D1 mRNA upregulation in response to PDGF-BB stimulation (Figure 2(a), $P < 0.05$). As shown in Figure 2(a), 100 nM dexamethasone significantly inhibited the amount of PDGF-BB-induced cyclin D1 mRNA expression at 8 h and 24 h after stimulation (Figure 2(a); $P < 0.05$), and a resultant attenuation of cyclin D1 protein expression was also observed at 24 h (Figure 2(b); $P < 0.05$).

In contrast, parallel experiments performed in ASM cells from asthmatics revealed that the PDGF-BB-induced upregulation of cyclin D1 mRNA and protein expression was resistant to inhibition by dexamethasone (Figure 3). As shown in Figure 3(a), there was no significant difference in the temporal kinetics of PDGF-BB-induced cyclin D1 mRNA expression in the presence or absence of dexamethasone. Moreover, there were no significant differences in amounts of PDGF-BB-induced cyclin D1 protein induced after pretreatment with dexamethasone (1.5 ± 0.2-fold), as compared to vehicle control (1.5 ± 0.2-fold) (Figure 3(b)).

To examine whether the degree of GR nuclear translocation differed in mitogen-treated ASM from asthmatics as compared to non-asthmatics, cells were pretreated with dexamethasone for 1 h, stimulated with PDGF-BB for 1 h, prior to preparation of purified nuclear and cytoplasmic extracts. As shown in Figure 4, dexamethasone induced the translocation of GR to a similar extent in cells from non-asthmatics (Figure 4(a)), compared to asthmatics (Figure 4(b)). PDGF-BB had no effect on the degree of GR nuclear translocation induced by dexamethasone.

4. Discussion

Increase in airway smooth muscle mass is a hallmark of the remodelled airway in asthma. Many studies have focused on the G_1-to-S transition, in particular the role of cyclin D1 in modulating G_1 progression to S-phase traversal in ASM cells from non-asthmatics. Our study examines cyclin D1 in ASM from asthmatic subjects, and compares the relative inhibitory efficacy of corticosteroids with ASM cells from non-asthmatics. We have confirmed that cyclin D1 upregulation in ASM cells is inhibited by corticosteroids [16]. We are the first to examine the effect of dexamethasone

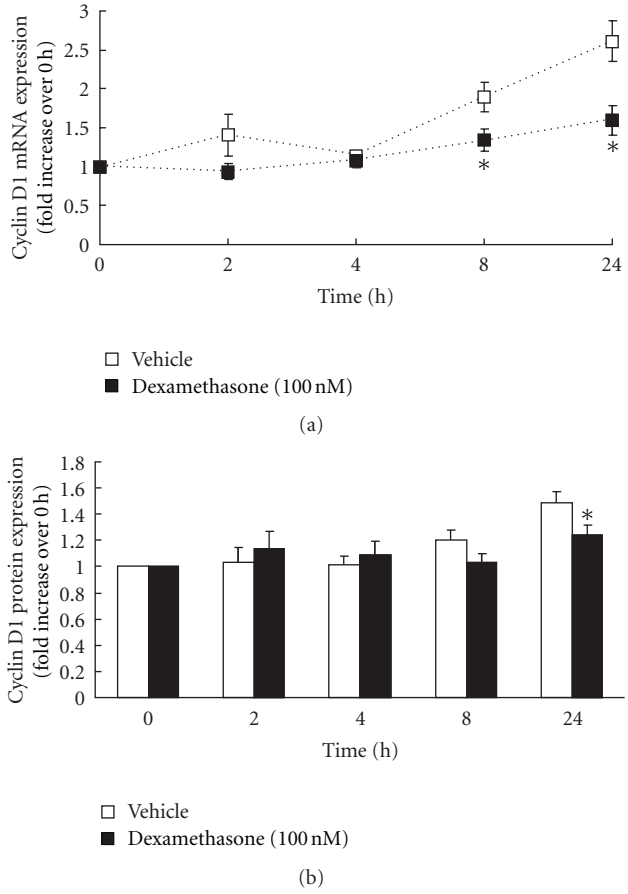

Figure 2: Dexamethasone inhibits cyclin D1 mRNA and protein expression in ASM cells from non-asthmatics. ASM cells from non-asthmatics ($n = 7 - 8$) were pretreated for 1 h with vehicle or dexamethasone and then stimulated with 25 ng/mL PDGF-BB. (a) demonstrates the effect of dexamethasone on the temporal kinetics of PDGF-BB-induced cyclin D1 mRNA expression (expressed as fold increase over 0 h). Statistical analysis was performed using two-way ANOVA, followed by Fisher's post hoc multiple comparison test (where $*$ denotes significant inhibition by dexamethasone ($P < 0.05$)). (b) shows densitometric analysis of the effect of dexamethasone on cyclin D1 protein (expressed as fold increase over 0 h), using α-tubulin as the loading control. Statistical analysis was performed using the Student's unpaired t-test (where $*$ denotes a significant inhibition by dexamethasone ($P < 0.05$)). Values are mean + SE.

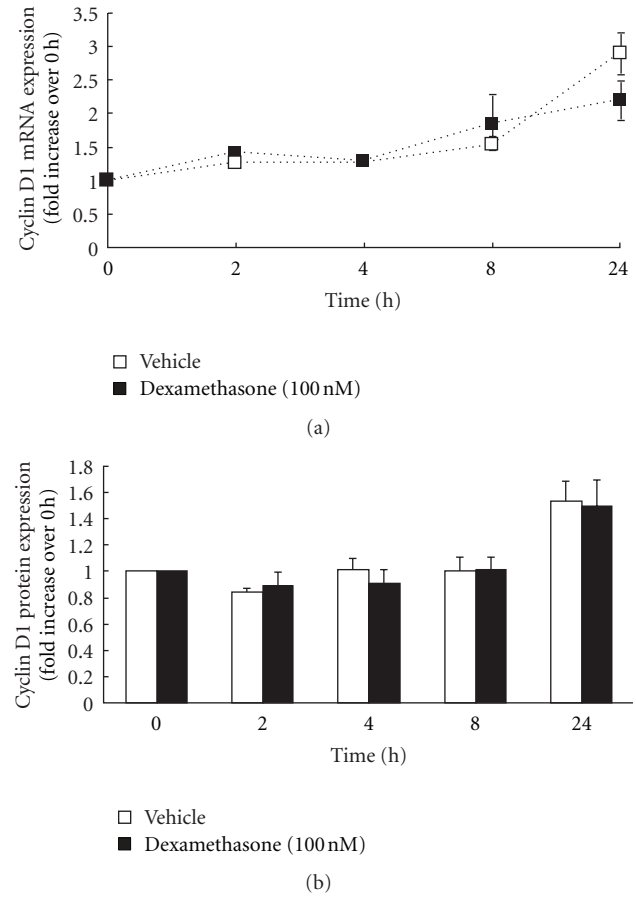

Figure 3: Cyclin D1 mRNA and protein expression in ASM cells from asthmatics were relatively resistant to inhibition by dexamethasone. ASM cells from asthmatics ($n = 7$) were pretreated for 1 h with vehicle or dexamethasone and then stimulated with 25 ng/mL PDGF-BB. (a) demonstrates the effect of dexamethasone on the temporal kinetics of PDGF-BB-induced cyclin D1 mRNA expression (expressed as fold increase over 0 h). (b) shows densitometric analysis of the effect of dexamethasone on cyclin D1 protein (expressed as fold increase over h), using α-tubulin as the loading control. Values are mean + SE.

on the mitogen PDGF-BB-induced cyclin D1 upregulation in ASM cells from both non-asthmatics and asthmatics. Intriguingly, we show that we are unable to totally inhibit cyclin D1 mRNA and protein upregulation in cells from asthmatic subjects. These results corroborate earlier evidence demonstrating that corticosteroid-inhibited proliferation occurs only in ASM cells from subjects without asthma [8] and suggest that there are corticosteroid insensitive proliferative pathways in asthmatics that warrant further investigation in order to design efficacious antiremodelling strategies.

How ASM mass increases in a remodelled airway is an area under intense investigation. *In vivo*, there is evidence of greater ASM cell number (hyperplasia) [17, 18]. *In vitro*, hyperplasia of ASM is well supported by numerous *in vitro* studies in which ASMs have been shown to proliferate in response to mitogenic inflammatory mediators present in the inflamed airways (reviewed in [4, 9, 10]), and by the observation that ASM cells from asthmatics have a greater rate of proliferation *in vitro* when compared to cells from non-asthmatics [13, 19]. Cyclin D1 upregulation, and its repression by corticosteroids, has been extensively examined in *in vitro* studies using cells from non-asthmatics (reviewed in [4, 9, 10]). Our study compares and contrasts the upregulation of cyclin D1, and the relative efficacy of corticosteroids, in cells from both asthmatics and non-asthmatics examined *in vitro*. We observe corticosteroid insensitivity in cells from asthmatics. This insensitivity was not due to altered nuclear translocation of the glucocorticoid receptor in ASM cells from asthmatics compared to non-asthmatics. Rather,

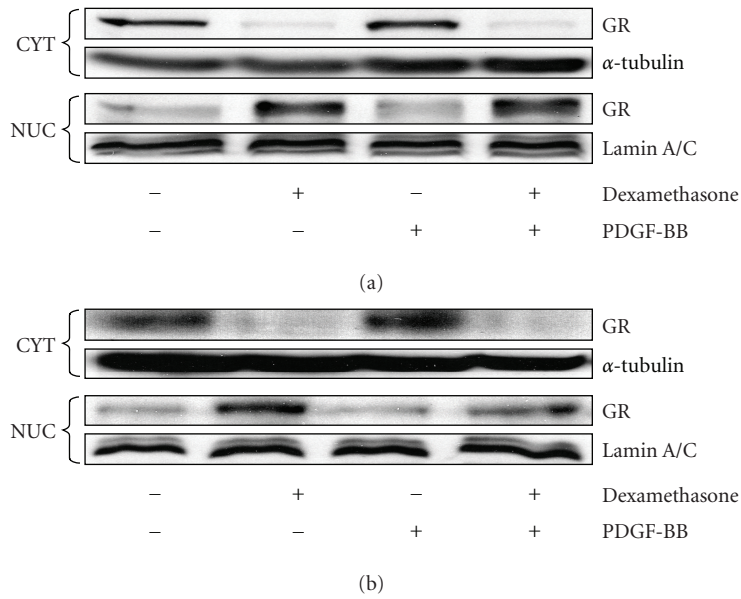

Figure 4: Dexamethasone-induced GR nuclear translocation does not differ between mitogen-treated ASM from asthmatics as compared to non-asthmatics. ASM cells from (a) $n = 3$ non-asthmatics or (b) $n = 3$ asthmatics were pretreated for 1 h with vehicle or dexamethasone (100 nM) and then stimulated for 1 h with 25 ng/mL PDGF-BB. Cytoplasmic (CYT) and nuclear (NUC) fractions were prepared, and GR protein was measured by western blotting (representative blots illustrated), using α-tubulin and lamin A/C as the loading controls for the cytoplasmic or nuclear fractions, respectively.

a number of studies may have posed some possible explanations for our observations. ASM cells are tethered within the extracellular-matrix- (ECM-) rich microenvironment of fibrotic asthmatic airway. In asthma, the abundance of ECM proteins, including collagen I, is increased [20]. Bonaccci et al. [21] demonstrated that integrin/collagen I interactions impaired the antimitogenic action of dexamethasone. As ASM cells from asthmatics secrete greater amounts of collagen I than non-asthmatics, integrin/ECM interactions may contribute to corticosteroid resistance in our experiments. As an alternative explanation, Roth et al. [8] demonstrated that the antiproliferative effect of corticosteroids in ASM cells requires the formation of a complex between the glucocorticoid receptor and the CCAAT/enhancer binding protein alpha (C/EBPα). As C/EBPα protein is absent in ASM cells from asthmatics, interaction of corticosteroid with its cognate receptor would be unable to form the required antimitogenic complex, explaining the failure of corticosteroids to inhibit cyclin D1-mediated proliferative pathways *in vitro*.

In summary, we have shown that cyclin D1 mRNA and protein upregulation in asthmatic ASM cells are insensitive to inhibition by corticosteroids. Our results may reflect the contribution of impaired corticosteroid action via ASM-ECM interactions or absence of a key transcriptional regulator of corticosteroid action (C/EBPα) in asthmatic cells. Which possibility is responsible for corticosteroid insensitivity in ASM cells from asthmatics is unknown at present, but this observation underscores the importance of investigations into underlying molecular mechanisms of ASM hyperplasia in asthma to allow future development of efficacious antiremodelling strategies as future asthma pharmacotherapeutics.

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Research Article

Combined Beta-Agonists and Corticosteroids Do Not Inhibit Extracellular Matrix Protein Production *In Vitro*

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Background. Persistent asthma is characterized by airway remodeling. Whereas we have previously shown that neither β_2-agonists nor corticosteroids inhibit extracellular matrix (ECM) protein release from airway smooth muscle (ASM) cells, the effect of their combination is unknown and this forms the rationale for the present study. *Methods*. ASM cells from people with and without asthma were stimulated with TGFβ1 (1 ng/ml) with or without budesonide (10^{-8} M) and formoterol (10^{-10} and 10^{-8} M), and fibronectin expression and IL-6 release were measured by ELISA. Bronchial rings from nonasthmatic individuals were incubated with TGFβ1 (1 ng/ml) with or without the drugs, and fibronectin expression was measured using immunohistochemistry. *Results*. Budesonide stimulated fibronectin deposition, in the presence or absence of TGFβ1, and this was partially reversed by formoterol (10^{-8} M) in both asthmatic and nonasthmatic cells. Budesonide and formoterol in combination failed to inhibit TGFβ-induced fibronectin in either cell type. A similar pattern of expression of fibronectin was seen in bronchial rings. TGFβ1-induced IL-6 release was inhibited by the combination of drugs. *Conclusion*. Current combination asthma therapies are unable to prevent or reverse remodeling events regulated by ASM cells.

1. Introduction

Airway remodeling, including alterations in the thickness of the basement membrane, an increase in the number of mucus producing cells, an increase in the number of blood vessels (angiogenesis), and a change in the extracellular matrix (ECM) protein profile and hypertrophy/hyperplasia resulting in an increase in the bulk of the airway smooth muscle (ASM), is now recognized as a hallmark feature of asthma. Little is known about the effectiveness of current asthma therapies upon these structural changes in the airways, particularly in the vicinity of the ASM.

We have previously reported that neither corticosteroids nor long-acting β_2-agonists (LABAs) alone are effective at preventing or reversing *in vitro* parameters of ASM-driven airway remodeling [1]. The critical question that remained was whether the combination of these two drug classes would be more effective.

Whilst the combination of inhaled corticosteroids and LABAs improves asthma control and lung function and decreases the frequency of asthma exacerbations compared to placebo or high doses of inhaled corticosteroids alone [2–6], few studies have examined their effectiveness at altering parameters of remodeling *in vivo*. One exception is the study by Orsida and colleagues who reported that the combination of LABAs and inhaled corticosteroids reduces blood vessel number [7]. Given our previous finding of the lack of effectiveness of these drugs singly in reducing parameters of airway remodeling, it was vital to assess their efficacy in combination.

Several studies have examined the *in vitro* effectiveness of combined corticosteroids and LABAs in fibroblasts with conflicting results. Goulet et al. found that corticosteroids and LABAs had opposing effects on matrix protein deposition in the presence of serum and their combination counteracted each other [8]. In contrast, also in fibroblasts, Descalzi et al. reported corticosteroids had significant antiproliferative effects and that combination with LABAs strengthened these effects [9]. Todorova et al. reported that corticosteroids reduced and the combination with LABAs

Table 1: Patient details.

Patient no.	Age (yrs)	Sex	Disease	Source of tissue	Sample type
1	76	Female	Chronic obstructive pulmonary disease	Endobronchial biopsy	ASM
2	43	Female	Asthma	Endobronchial biopsy	ASM
3	26	Male	Asthma	Endobronchial biopsy	ASM
4	66	Male	Chronic obstructive pulmonary disease	Resection	ASM
5	22	Male	Asthma	Endobronchial biopsy	ASM
6	38	Female	Carcinoid (atypical)	Resection	ASM
7	46	Female	Carcinoma	Resection	ASM
8	58	Male	Emphysema	Explanted lungs	ASM
9	50	Female	Asthma	Endobronchial biopsy	ASM
10	56	Female	Emphysema	Explanted lungs	ASM
11	40	Male	Asthma	Endobronchial biopsy	ASM
12	27	Male	Asthma	Endobronchial biopsy	ASM
13	68	Female	Carcinoma	Resection	ASM
14	55	Male	Emphysema	Explanted lungs	ASM
15	64	Female	Emphysema	Explanted lungs	ASM
16	21	Male	Asthma	Endobronchial biopsy	ASM
17	25	Female	Bronchiolitis Obliterans	Explanted lungs	Bronchial rings
18	48	Female	Emphysema	Explanted lungs	Bronchial rings

ASM: airway smooth muscle.

abolished proteoglycan production induced by serum [10]. In the absence of serum, regardless of whether transforming growth factor β (TGFβ) was present or not, fluticasone increased fibronectin at both the mRNA and protein levels; however, it decreased tenascin-C at both levels. Salmeterol did not affect fibronectin or tenascin-C nor did it alter the effect of fluticasone when the drugs were applied in combination [11].

Whilst we, and others, have begun elucidating the molecular mechanism underlying the synergistic effect of the combination of corticosteroids and LABAs in ASM cells [12, 13], the effect of the combined drugs on the release of ECM proteins from ASM cells remains to be investigated.

In this study, we hypothesized that the combination of corticosteroids and LABAs would be ineffective at inhibiting the production of ECM proteins *in vitro*. To investigate this hypothesis, we examined the effect of the combination of corticosteroids and LABAs in a well-characterized model of *in vitro* airway remodeling [1], namely, TGFβ-induced fibronectin in human asthmatic and nonasthmatic ASM cells *in vitro* and in nonasthmatic bronchial rings *ex vivo*.

2. Materials and Methods

2.1. Cell Culture. Approval for all experiments with human lung was provided by the Human Ethics Committees of The University of Sydney and the Sydney South West Area Health Service. Asthmatic ASM was obtained from 7 patients (mean age 32.7 ± 11.5 years SD) either undergoing resection for lung transplantation or deep endobronchial biopsies. Nonasthmatic ASM was obtained from bronchial airways of 9 patients (mean age 58.6 ± 11.6 years SD) undergoing resection for either lung transplantation or carcinoma. The characteristics of the patients are listed in Table 1. Pure ASM bundles were dissected free and grown as explants as previously described [13–15]. ASM cell characteristics were determined by light microscopy and immunofluorescence for the detection of α-smooth muscle actin and calponin [16]. All experiments were performed with cells between passages 4 and 8.

2.2. Airway Smooth Muscle Cell Treatment. ASM cells from 6 asthmatic and 8 nonasthmatic patients were seeded for 24 hours in 5% fetal bovine serum (FBS) (JRH Biosciences, Melbourne, Australia) Dulbecco's Modified Eagle's Medium (DMEM) (SAFC Biosciences, Lenexa, KS) in the presence of 20 U/mL penicillin, 20 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Invitrogen, Heidelberg, Australia) at a density of 1×10^4 cells per cm^2. Medium was then changed to 0.1% insulin transferrin selenium (ITS) (Invitrogen, Heidelberg, Australia) DMEM for 24 hours before addition of formoterol (0.1 and 10 nmol/L) and budesonide (0.1 and 10 nmol/L) alone or in combination as indicated 30 minutes prior to stimulation with TGFβ1 (1 ng/mL) for the time periods described below. The effect of the drugs in unstimulated cells was assessed by omission of the TGFβ stimulation in cells maintained in 0.1% ITS. All of the drugs were dissolved in aqueous solutions.

2.3. ELISAs

2.3.1. Deposited ECM Protein ELISAs. ASM cells from 6 asthmatic and 6 nonasthmatic patients were seeded in 96 well plates and treated as described above for 48 hours. ECM free of cells was prepared by treatment with sterile hypotonic ammonium hydroxide [17–19]. Fibronectin was measured by ELISA as previously described [19] using an antibody to fibronectin (mouse antihuman plasma fibronectin 2 μg/mL,

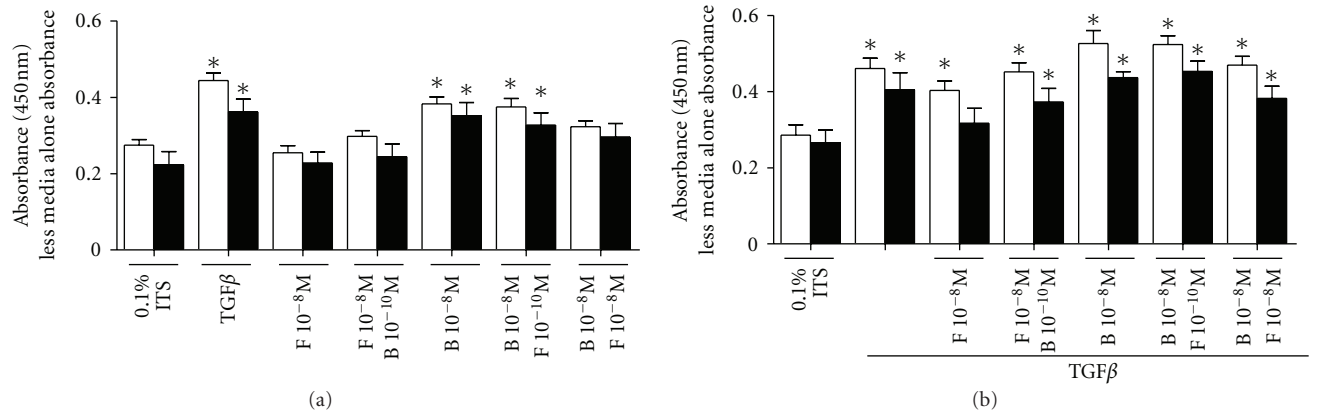

Figure 1: Effect of combined corticosteroids and LABAs on the deposition of fibronectin in the absence (a) or presence of TGFβ (b) for 48 hrs, respectively. Data are mean ± SEM from $n = 6$ asthmatic (black bars) and nonasthmatic (white bars) ASM cell lines. *Significantly different from nondrug-treated control $P < 0.05$. F: formoterol, B: budesonide.

clone 868A11, Chemicon, Temecula CA) and a purified mouse IgG_1 isotype control 2 μg/mL, clone MOPC-31C, (Becton and Dickinson Pharmingen, San Jose, CA).

2.3.2. IL-6 ELISAs. ASM cells from 6 asthmatic and 8 nonasthmatic patients were seeded in 24 well plates and treated as described above for 48 hours. Supernatants were collected in aliquots and stored at −20°C until analysis. IL-6 release was detected using an IL-6 ELISA kit according to the manufacturer's instructions (Duoset, Becton and Dickinson, San Jose, CA).

2.3.3. Soluble Fibronectin ELISAs. Supernatants collected as described above were also assayed for soluble fibronectin release using a Quantimatrix Human fibronectin ELISA kit according to the manufacturer's instructions (Chemicon International, Temecula, CA).

2.3.4. Immunohistochemistry. Human lung tissue was obtained from lung specimens resected for carcinoma or transplantation. Bronchial rings (2–5 mm diameter and 3 mm in length) were dissected free from surrounding parenchymal tissue. The bronchial rings were incubated in treatments as described above. After 24 hours, tissues were frozen in optimal cutting temperature (OCT) embedding medium (Fronine Laboratory Supplies, Riverstone, Australia), sectioned on a cryostat and immunohistochemistry performed using mouse anti-fibronectin (1 μg/mL Chemicon International, Temecula, CA) coupled with a horseradish peroxidase labeled polymer. To help identify the morphology of the tissue, hematoxylin and eosin (H&E) staining was performed on adjacent sections. Full details of this method have been described previously [1].

2.3.5. Analysis of Data. For ECM ELISA data, results from duplicate wells from each individual subject were averaged and the absorbance from media alone subtracted before an overall mean and standard error of the mean (SEM) were obtained from asthmatic and nonasthmatic cells. Analysis of variance (ANOVA) repeated measures with bonferonni post-tests or student's paired t-tests were performed on the results for ECM ELISAs where appropriate. In all cases a P value of less than or equal to 0.05 was considered significant.

3. Results

3.1. Effect of Combined Corticosteroids and LABAs on Basal ECM Protein Deposition. Budesonide alone (10^{-8} M) induced fibronectin deposition in both asthmatic and nonasthmatic ASM cells (Figure 1), in agreement with our previous study [1]. The addition of formoterol (10^{-8} M but not 10^{-10} M) abolished the induction of fibronectin by budesonide 10^{-10} M and 10^{-8} M (Figure 1(a) and Table 2).

3.2. Effect of Combined Corticosteroids and LABAs on TGFβ Stimulated ECM Protein Deposition. TGFβ induced the deposition of fibronectin from both asthmatic and nonasthmatic ASM cells, in agreement with our previous reports [1, 20, 21] (Figure 1). The addition of formoterol (10^{-10} M and 10^{-8} M) or budesonide (10^{-8} M or 10^{-10} M), alone or in combination, did not significantly alter fibronectin deposition in the presence of TGFβ in either cell type.

3.3. Effect of Combined Corticosteroids and LABAs on TGFβ Stimulated Soluble Fibronectin Release. The release of soluble fibronectin from asthmatic and nonasthmatic ASM cells was increased by TGFβ but the presence of the drugs, in any combination, did not alter the release of fibronectin (data not shown).

3.4. Effect of Combined Corticosteroids and LABAs on Basal IL-6 Release. In nonasthmatic ASM cells, budesonide (10^{-8} M) alone significantly reduced the release of IL-6. The addition of formoterol (10^{-10} or 10^{-8} M) did not reverse this reduction (Figure 2(a) and Table 3). The release of IL-6 from asthmatic ASM cells was more variable but followed the same pattern.

Table 2: Effect of combined corticosteroids and LABAs on basal and TGFβ-stimulated ECM protein deposition.

Asthmatic			Alone		Nonasthmatic		Alone	
		—	F 10^{-10} M	F 10^{-8} M		—	F 10^{-10} M	F 10^{-8} M
Alone	—			104.8 ± 6.7	—			92.3 ± 2.5
	B 10^{-10} M			111.5 ± 9.2	B 10^{-10} M			108.5 ± 2.1
	B 10^{-8} M	166.4 ± 13.4*	154.8 ± 13.0	137.1 ± 8.3	B 10^{-8} M	140.5 ± 7.4*	137.3 ± 7.7	117.9 ± 4.4
			TGFβ stimulated				TGFβ stimulated	
		—	F 10^{-10} M	F 10^{-8} M		—	F 10^{-10} M	F 10^{-8} M
TGFβ stimulated	—			78.3 ± 2.5*	—			87.4 ± 2.0*
	B 10^{-10} M			93.6 ± 3.8	B 10^{-10} M			98.5 ± 3.6
	B 10^{-8} M	113.5 ± 10.2	116.0 ± 8.1	97.1 ± 6.5	B 10^{-8} M	114.1 ± 3.0*	114.2 ± 2.8	102.3 ± 2.7

F: formoterol, B: budesonide.
Data are expressed as % 0.1% ITS for drugs alone and % TGFβ for TGFβ-stimulated samples.
*significantly diff to 0.1% ITS or TGFβ $P < 0.05$. $n = 6$ asthmatic and 6 nonasthmatic.

Table 3: Effect of combined corticosteroids and LABAs on basal and TGFβ-stimulated IL6 release.

Asthmatic			Alone		Nonasthmatic		Alone	
		—	F 10^{-10} M	F 10^{-8} M		—	F 10^{-10} M	F 10^{-8} M
Alone	—			275.6 ± 71.4	—			224.0 ± 68.2
	B 10^{-10} M			208.3 ± 54.8	B 10^{-10} M			181.5 ± 51.6
	B 10^{-8} M	31.9 ± 12.7	54.1 ± 22.5	166.6 ± 48.7	B 10^{-8} M	23.35 ± 2.5	78.13 ± 47.5	187.1 ± 112.7
			TGFβ stimulated				TGFβ stimulated	
		—	F 10^{-10} M	F 10^{-8} M		—	F 10^{-10} M	F 10^{-8} M
TGFβ stimulated	—			68.2 ± 9.2	—			74.1 ± 9.5
	B 10^{-10} M			55.8 ± 7.9	B 10^{-10} M			58.78 ± 5.4
	B 10^{-8} M	13.7 ± 3.6	13.7 ± 4.0	21.1 ± 5.5	B 10^{-8} M	20.0 ± 1.9	17.3 ± 2.9	17.6 ± 2.9

F: formoterol, B: budesonide.
Data are expressed as mean ±SEM % 0.1% ITS for drugs alone and % TGFβ for TGFβ-stimulated samples. $n = 6$ asthmatic and 8 nonasthmatic.

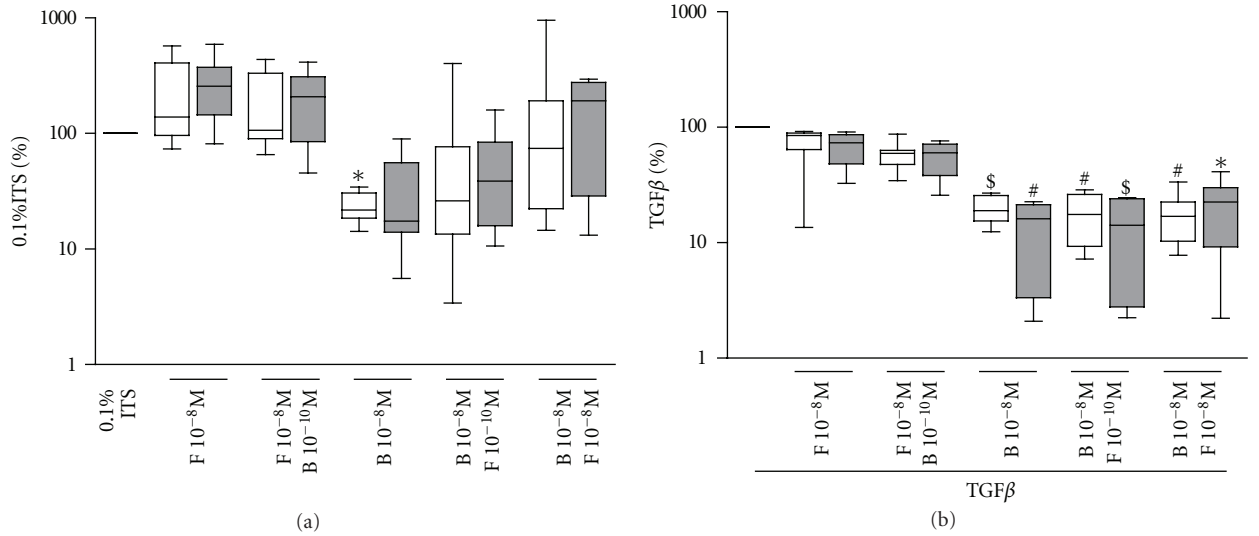

Figure 2: Effect of combined corticosteroids and LABAs on the release of IL-6 in the absence (a) or presence of TGFβ (b) for 48 hrs respectively. Data are mean ± SEM from $n = 6$ asthmatic (grey boxes) and $n = 8$ nonasthmatic (white boxes) ASM cell lines. Significantly different from nondrug-treated control $^{*}P < 0.05$, $^{\#}P < 0.005$, $^{\$}P < 0.001$. F: formoterol, B: budesonide.

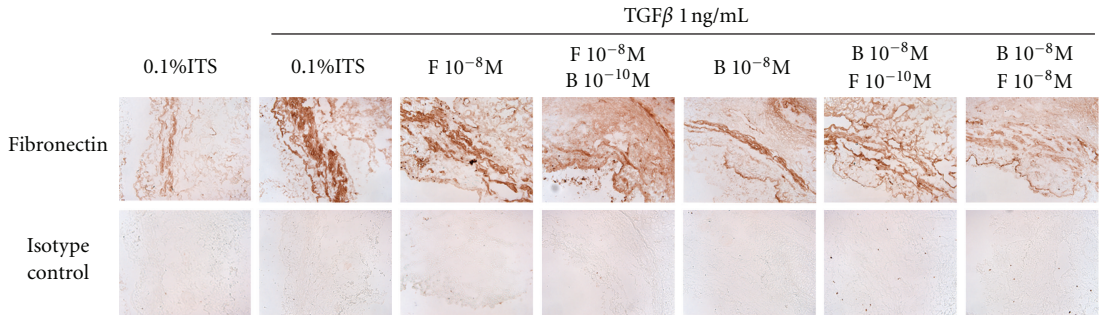

Figure 3: Effect of combined corticosteroids and LABA on TGFβ-induced fibronectin in nonasthmatic bronchial rings. Immunohistochemical detection of fibronectin (brown staining) basally or following stimulation with TGFβ in the presence or absence of drugs in nonasthmatic tissue sections.

3.5. Effect of Combined Corticosteroids and LABAs on TGFβ Stimulated IL-6 Release. In both cell types, TGFβ significantly induced the release of IL-6. Budesonide reduced the release of IL-6 even in the presence of TGFβ in both cell types (Figure 2(b) and Table 3). Once again, formoterol (10^{-10} and 10^{-8} M) did not reverse the inhibitory effect of budesonide (asthmatic 13.66 ± 4.0 and 21.08 ± 5.5, nonasthmatic 17.34 ± 2.9 and 17.55 ± 2.9% of TGFβ) (Figure 2(b)).

3.6. Effect of Combined Corticosteroids and LABAs on Fibronectin Expression in Bronchial Tissue Rings. To examine the effectiveness of the combination of corticosteroids and LABAs on ECM deposition in the whole airway, we used our *ex vivo* bronchial ring model [1, 20]. Bronchial rings from two nonasthmatic individuals stimulated with TGFβ showed increased deposition of fibronectin, in agreement with our previous findings [1, 20]. Neither formoterol nor budesonide alone, or in combination, reduced the TGFβ-induced fibronectin deposition (Figure 3).

4. Discussion

Our previous work demonstrated that neither long-acting beta agonists nor corticosteroids reduced the release of ECM proteins from ASM cells. The question remained as to whether the combination of these two therapeutic drug classes might be more effective. The results of the current study with formoterol and budesonide demonstrate that this is not the case, regardless of whether the cells were derived from asthmatic or nonasthmatic subjects. Moreover, in bronchial rings stimulated with TGFβ, fibronectin deposition was not reduced by formoterol, budesonide, nor their combination.

There are many examples of the efficacy of combined LABAs and corticosteroids in both *in vivo* [5, 22] and *in vitro* [13, 23–25] studies. There are very few reports, however, of the modulation of remodeling parameters by these drugs, although the combination of LABAs and inhaled corticosteroids does reduce angiogenesis—one of the features of remodeling [7]. In addition, we have reported a synergistic inhibition of ASM proliferation when these drugs are studied in combination [13]. However, budesonide and salbutamol, alone or in combination, had no effect on collagen fiber tractional remodeling as ASM cells migrated through collagen gels [26]. Descalzi et al. [9] found that the combination of beclomethasone dipropionate (BDP) with either a short or long-acting beta agonist decreased fibronectin production induced by basic fibroblast growth factor, and that this effect was greater than with BDP alone. Their study was carried out in fibroblasts stimulated with basic fibroblast growth factor, as opposed to smooth muscle cells stimulated with TGFβ in the current study, and this may be the basis for the differences observed. Again, in fibroblasts, corticosteroids in the presence of serum increased ECM deposition, which we also found in ASM cells, but LABAs decreased ECM deposition and the net result of the combination was simply additive [8]. In contrast, Degen et al. found, in fibroblasts, that fluticasone increased fibronectin but decreased tenascin-C mRNA and protein induced by FBS, TGFβ, or in the absence of stimulation. Under these experimental conditions salmeterol did not influence the fluticasone effects [11]. We also examined the effect of corticosteroids and LABAs alone and in combination on TGFβ-induced soluble, as opposed to matrix-associated, fibronectin release but again these interventions were without effect in either asthmatic or nonasthmatic cells. To our knowledge, there are no previous reports examining the effect of combination therapy in asthmatic ASM.

A consistent finding from our laboratory has been the increase in release of ECM proteins from ASM in response to corticosteroids. Beclomethasone increased release of fibronectin from ASM [19] and this effect has also been reported by Goulet et al. and Degen et al. in human airway fibroblasts using several corticosteroids [8, 11]. This is consistent with the fact that budesonide increased fibronectin release from ASM cells derived from both asthmatic and nonasthmatic subjects in our current study, and furthermore this occurred whether or not cells were stimulated with TGFβ. Interestingly, formoterol was able to attenuate budesonide-induced ECM fibronectin deposition even though alone it was without effect. The differential response of fibroblasts to fluticasone in relation to the production of fibronectin and tenascin-C observed by Degen et al. [11] suggests that the individual ECM proteins may respond

differently to therapeutic intervention. Therefore, caution should be taken in interpreting the results of this *in vitro* study as a global representation of the effectiveness of current therapies on altering parameters of airway remodeling.

Although we found in the present study that the combination of LABAs and corticosteroids did not decrease fibronectin release, corticosteroids, as previously reported [1, 27], inhibited IL-6 release from the ASM cells. Baouz et al. reported that in (myo) fibroblasts, salmeterol inhibited IL-6 release, and this was amplified by the addition of low concentrations of fluticasone dipropionate [28]. Others have found, also in fibroblasts, that corticosteroids inhibited and LABAs had no effect on IL-6 release and the effect of the combination was that of corticosteroids alone [8]. In contrast, IL-6 release from ASM is increased by β_2-agonists in both asthmatic [1] and nonasthmatic cells [1, 29], and our findings in the current study confirm this.

The study of cells in culture is associated with limitations, and this is where we find the bronchial ring preparation a useful model. It enables us to observe, in an "intact" airway, changes in ECM proteins [1, 17] and cytokine deposition [15, 30] in response to profibrotic stimuli such as TGFβ and, in addition, to investigate the effects of intervention with relevant therapeutic agents such as LABAs and corticosteroids. Here we confirmed our previous findings [1] that neither LABAs nor corticosteroids alone decreased fibronectin deposition in response to TGFβ and extended them to include the combination of the two drug classes which were without effect. In contrast, we have previously reported that the phosphodiesterase inhibitor roflumilast abolished TGFβ-induced fibronectin deposition [1].

In summary, in our cell and tissue models of ECM protein deposition, we investigated whether the combination of a LABA and a corticosteroid would be more effective in inhibiting or reversing TGFβ-induced fibronectin release. This was not the case either in cells derived from asthmatic or nonasthmatic volunteers, or in intact bronchial rings. Airway remodeling is detrimental in the pathophysiology of asthma, and ECM protein deposition is a major component of said remodeling; therefore, these results highlight the need for further development of agents to reverse or prevent parameters of airway remodeling.

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Review Article

How Can Microarrays Unlock Asthma?

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Asthma is a complex disease regulated by the interplay of a large number of underlying mechanisms which contribute to the overall pathology. Despite various breakthroughs identifying genes related to asthma, our understanding of the importance of the genetic background remains limited. Although current therapies for asthma are relatively effective, subpopulations of asthmatics do not respond to these regimens. By unlocking the role of these underlying mechanisms, a source of novel and more effective treatments may be identified. In the new age of high-throughput technologies, gene-expression microarrays provide a quick and effective method of identifying novel genes and pathways, which would be impossible to discover using an individual gene screening approach. In this review we follow the history of expression microarray technologies and describe their contributions to advancing our current knowledge and understanding of asthma pathology.

1. Introduction

Asthma is a complex chronic inflammatory disease which affects ~300 million individuals worldwide, causing an estimated economic cost of $19.7 billion in direct and indirect costs each year [1, 2]. Asthma can be defined by a number of characteristics, including (1) airway hyperresponsiveness (AHR), (2) airway remodeling, and (3) airflow obstruction including bronchoconstriction, mucus plugging, and inflammation [3]. The presence and severity of these characteristics can be influenced by many factors including age, ethnicity, gender, genetic predisposition, and the environment [4–7]. The asthma phenotype is further confounded by the existence of possible subtypes of asthma, which go beyond the common mild, moderate, and severe groupings [8]. This heterogeneity has thus far been a major hindrance in the search for susceptible genes for asthma, and it is becoming increasingly apparent that asthma is the result of dysregulation of a number of complex pathways instead of any single gene. In a new age of high-throughput technologies, gene-expression microarrays provide a quick and effective method of identifying novel genes and pathways which would be impossible to discover using an individual gene screening approach. Programs used to analyse and identify significant pathways based on microarray data have previously been reviewed [9] and will not be discussed here. In this review we will follow the history of gene-expression microarray technologies and describe their contributions to our current understanding of asthma pathology.

2. Methods for Identifying Disease Causing Genes

Since early evidence for a genetic component for asthma was most strongly demonstrated by a higher concordance for asthma among monozygotic than dizygotic twins [10], the search for genes influencing this disease has relied on three main approaches: genomewide association studies (GWASs)/locus fine mapping, gene candidate approaches, and gene expression studies (gene-expression microarrays). The first two methods have been extensively reviewed [11, 12] and therefore will only be briefly mentioned here. GWASs have been essential in the discovery of many asthma-associated genes including disintegrin and metalloproteinase

domain-containing protein 33 (ADAM33) (the extensively studied gene thought to be involved with airway remodeling), inactive dipeptidyl peptidase 10 (DPP10), neuropeptide S receptor 1 (NPSR1), histocompatibility antigen, class I, G (HLA-G), and PHD finger protein 11 (PHF11) [12–16]. GWASs rely on the variation of genes or surrounding DNA which occurs between individuals and uses this variation to measure the probability that certain single nucleotide polymorphisms (SNPs) (changes to the DNA sequence which may result in changes to the amino acid sequence of a protein) are linked to a disease. Because no prior knowledge of gene function is required, GWASs are considered an unbiased technique. In contrast, the gene candidate approach only looks at a specific region of the genome within or surrounding a gene of interest.

Gene-expression microarrays provide a platform to measure and compare the expression level of all genes within a genome at a single point in time. This platform therefore allows users to identify genes/microRNAs (miRNAs) which may be up/downregulated when comparing different types of tissue (e.g., diseased versus normal) or stimulations with certain drugs (treated versus untreated). Like GWAS, gene-expression microarrays are considered an unbiased technique allowing for the identification of truly novel genes. Furthermore gene-expression microarrays provide a tool to genetically profile diseases, helping to separate diseases into subtypes or predict the outcome of certain treatments. Despite numerous advantages, the use of gene-expression microarrays in asthma research is still in its infancy.

3. Macroarrays: Where It All Began

Macroarrays were the predecessors to the current day gene-expression microarray; they had the ability to test anywhere between 500 and 18000 cDNA transcripts, which were usually spotted onto a nylon membrane by an arrayer (a device connected to a computer allowing for precise placing and cataloguing of samples on an array) (Figure 1) [17]. The cDNA spotted onto macroarrays was obtained from bacterial libraries, which were developed by inserting total human transcripts into bacteriophage vectors and transfecting these vectors into bacteria, usually *Escherichia coli*. These vector carrying bacteria were grown and pure colonies were sequenced and amplified by PCR prior to being spotted on a macroarray.

Target transcripts for macroarrays were usually radioactively labeled by reverse-transcribing sample RNA with ^{33}phosphate-deoxyribonucleotide triphosphates (^{33}P-dNTPs). Samples were then hybridized to the spotted macroarray and quantified by measuring the amount of radio-emission from each spot. Differential gene expression was calculated by comparing the emission intensity of samples spotted on to duplicate macroarrays. Despite being the groundbreaking technology of their day, macroarrays had a number of problems. The main limitations of macroarrays were the low density of probes per array (fewer genes could be investigated per array), the large volumes of sample required for hybridization (up to 50 mL compared with 200 μL used for current gene-expression microarrays), and the reliability of the bacterial libraries. In some cases the bacterial libraries were not composed of pure colonies (not all bacteria in a single spot contained the same cDNA insert) making it difficult to determine which transcript was represented by a particular spot on the macroarray.

4. Gene-Expression Microarrays

Microarrays were the next step forward in the evolution of gene expression studies, with the advances in array technology being pioneered by Patrick Brown's laboratory [18]. Microarrays, unlike their predecessor, were spotted onto glass slides allowing for a higher density of probes (decreasing the amount of sample required to interrogate the same number of genes) and no longer used radio-actively labeled nucleotides. These arrays were created using a precise *xyz* robot that was programmed to spot cDNA samples on the substrate in precise locations to allow identification of genes with expression changes during the analysis phase of the experiment [18]. A number of technologies have been released using this platform including the dual color microarray (or two-color microarray) process explained in Figure 2 and these have been reviewed previously [17]. An alternative technology for the production of microarrays was developed using photolithographic masks to create templates to enable *in situ* synthesis of oligonucleotides (usually 20–30 bps) directly on the glass substrate. Affymetrix pioneered the use of this platform of array production with the development of their "GeneChip" series of arrays, and in this review we will focus on the 3′ *in vitro* transcription (IVT) Expression GeneChip, as the majority of asthma-related studies have been conducted using this platform; however there are a large range of other expression microarrays produced by other companies which have been previously reviewed [19].

5. 3′ IVT Expression Microarrays

The 3′ IVT array microarray is historically the most common platform used by researchers conducting gene-expression microarray experiments in the asthma research field. The initial asthma gene-expression microarray studies using human cells were conducted in 2001 on the Affymetrix Hugene FL microarray containing probes representing ~6,500 human genes from the UniGene Build 18, GenBank, and the Institute for Genomic Research (TIGR) databases (Table 1). As gene-expression microarray technology advanced and mRNA databases became more complete, further versions of this platform were released, increasing the number, specificity, and annotation of the microarray probes with each subsequent release (Table 1). In 2004, the asthma community turned to the Affymetrix GeneChip 95A, the successor for the Affymetrix Hugene FL microarray, containing probes for ~12,000 full-length genes, derived from sequences in UniGene Build 95A (created from GenBank 113 and dbEST/10-02-99), including all the sequences represented on the Hugene FL microarray (Table 1).

In recent years, Affymetrix has released the Affymetrix GeneChip Human Genome U133 (HG-U133) containing

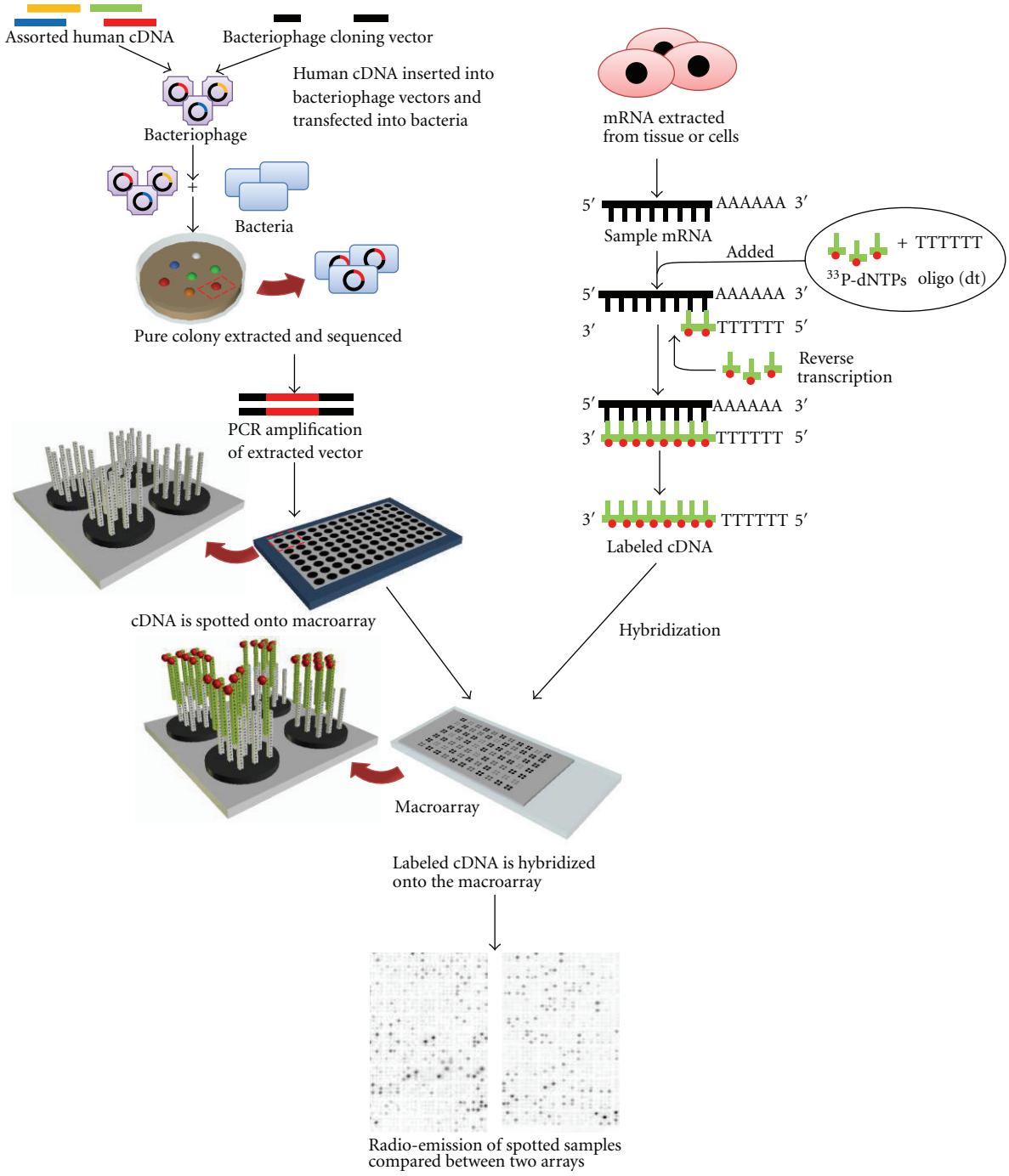

Figure 1: Overview of the production and use of Macroarrays. Macroarrays were constructed from cDNA held within bacterial libraries. These libraries were developed by inserting total human cDNA into bacteriophage vectors and transfection into bacteria. Pure colonies of bacteria carrying vectors were sequenced and amplified by PCR prior to spotting on to a macroarray. Samples were labeled by reverse-transcribing mRNA with radioactively labeled ^{33}phosphate-deoxyribonucleotide triphosphates (^{33}P-dNTPs) using specific oligo(dT) primers. Labeled cDNA samples were hybridized to duplicate macroarrays where gene expression was quantified by comparing the radio-emissions of each spot.

probes representing ~33,000 genes (created from GenBank, dbEST, and RefSeq) followed by their most recent version, the Affymetrix GeneChip Human Genome U133 Plus 2.0 array, which contains all the probes from its previous version plus those for 6,500 new genes (Table 1).

5.1. Preparing Samples for Analysis on the 3′ IVT Expression GeneChip. Although many versions of the 3′ IVT array have been released, the methods for preparing samples for these microarrays remain mostly unchanged. To prepare the samples for the 3′ IVT array, mRNA is first extracted

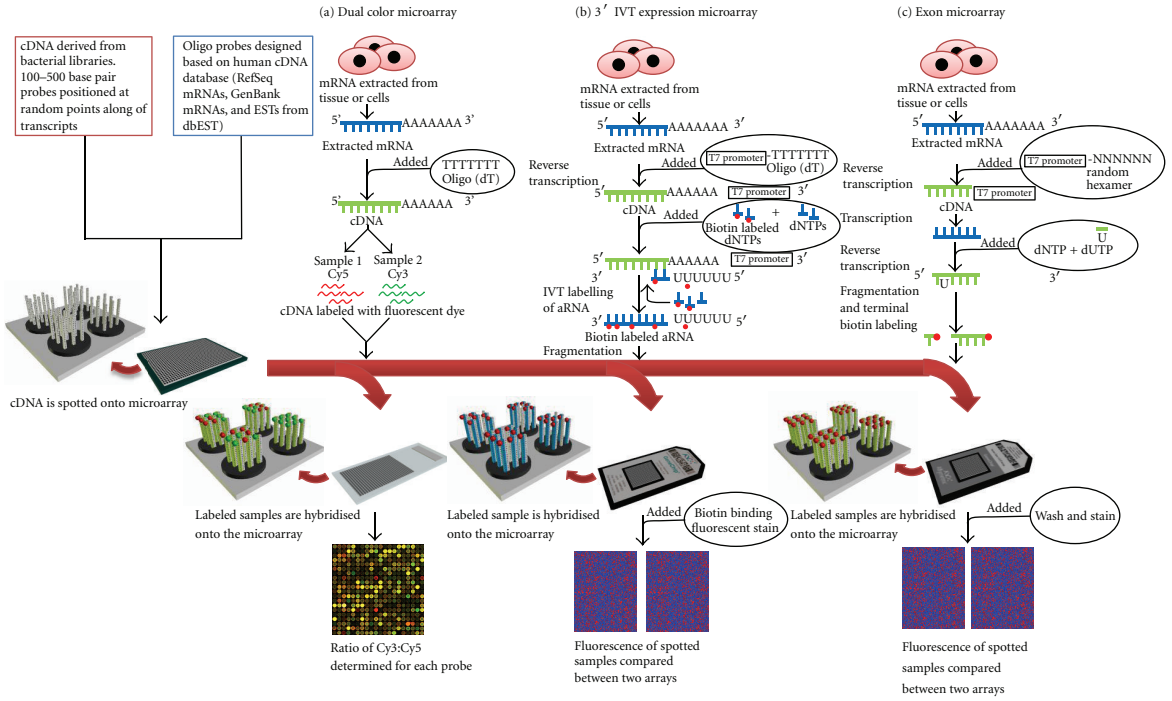

Figure 2: Overview of the production and use of expression microarrays. 3′ Expression arrays use synthetically derived oligo probes with design based on mRNA Databases (RefSeq mRNAs, GenBank mRNAs, and ESTs from dbEST) or cDNA derived from bacterial libraries (see Figure 1). Sample mRNA can be labeled using two methods (a) Cy3/Cy5 labeling: sample mRNA is reverse transcribed into cDNA and Cy3 is added to one sample and Cy5 to another. Both labeled samples are hybridized to the same microarray. (b) 3′ IVT array: sample mRNA is reverse transcribed to cDNA using oligo(dT) primers, to provide a template for transcription. Using biotin-conjugated nucleotides, the template cDNA is then converted to amplified RNA (aRNA). The biotin-labeled aRNA samples are then fragmented and hybridized onto 3′ expression arrays. A biotin binding fluorescent stain is added to the microarray after hybridization. (c) Affymetrix HuExon 1.0 ST: sample mRNA is reverse transcribed to cDNA using random primers, to provide a template for transcription. The resulting RNA is then reverse transcribed in the presence of dUTPs which are incorporated occasionally into the cDNA sequence instead of dTTP. An enzyme is then used to cleave the cDNA at the site of dUTP incorporation and fragments are terminally labeled before hybridization onto the array. The microarray is then washed and stained after hybridization.

from the targeted sample and converted to cDNA via reverse transcription using oligo(dT) primers attached to a T7 promoter (Figure 3). Oligo(dT) primers are short strings of dTs which selectively bind to the poly-A tails (of mRNA). Although this process was quite successful in binding to the majority of mRNAs, transcripts without poly-A tails (non-polyadenylated) were lost during this step. Current technology for the purpose of priming for reverse transcription uses random hexamers (strings of six random dNTPs) which capture sequences at any location along a transcript. This will be discussed later (see Section 8).

The cDNA is then converted to double stranded DNA (using the T7 promoter), to provide a template for transcription. Using biotin-conjugated nucleotides, the template DNA is then converted to amplified RNA (aRNA). The biotin-labeled aRNA samples are then fragmented and hybridized onto 3′ expression arrays and visualized by staining with phycoerythrin.

5.2. 3′ IVT Expression GeneChip Probes. Unlike the macroarrays previously described and a number of other gene-expression microarrays available on the market, 3′ IVT Expression GeneChips do not use cDNA libraries spotted onto an array. Instead Affymetrix arrays use short (~25 bp) nucleotide probes synthesized directly on the array; this process is well explained in a previous review [17]. Gene expression is determined by the hybridization of transcripts to perfect match (PM) and mismatch (MM) probes. Transcripts will preferentially bind to PM probes as they provide a perfect complimentary sequence to their matching transcript. MM probes are designed to resemble PM probes but differ (change in a single nucleotide) just enough for the target transcript not to bind. Therefore any transcripts binding to these MM probes are considered to represent background hybridization; by the use of the function (PM hybridization, MM hybridization) background hybridization can be calculated and taken into account. However, the use of MM probes to identify background binding has been slowly phased out because of a variety of technical reasons including the occurrence of "negative" expression levels when expression is low and the MM intensity exceeds the PM. For example, the R-Bioconductor preprocessing pipelines frequently omit MM probes [20].

Table 1: Databases used in Affymetrix microarray annotation.

Database	Description	Website	References
Expressed Sequence Tag Database (dbEST)	Division of GenBank that contains "single-pass" cDNA sequences (only sequenced once), or "Expressed Sequence Tags"	http://www.ncbi.nlm.nih.gov/dbEST/	[78]
The Institute for Genomic Research (TIGR)	Constructed by clustering, then assembling expressed sequence tag (EST) and annotated gene sequences from GenBank	http://compbio.dfci.harvard.edu/tgi/	[79]
UniGene Build	Contains transcript sequence information including: protein similarities, gene expression, cDNA clone reagents, and genomic location	http://www.ncbi.nlm.nih.gov/unigene/	[80]
GenBank	Annotated collection of all publicly available DNA sequences	http://www.ncbi.nlm.nih.gov/genbank/	[81]
The Reference Sequence (RefSeq)	Contains nonredundant, and well-annotated genomic DNA, transcripts, and protein sequences	http://www.ncbi.nlm.nih.gov/RefSeq/	[82]

6. 3′ Expression Arrays: Influence on Asthma Research

3′ Expression arrays have played a key role in asthma research through the screening for, and identification of, genes which are affected by asthma relevant stimuli and the direct comparison of asthmatic tissue to nonasthmatic tissue. Initial studies for asthma using the 3′ platform focused on identifying key cell types which play a role in asthma; therefore many researchers conducted their studies on human-isolated cells expanded in culture. One of the first isolated cell gene-expression microarray studies was conducted by Lee and group in 2001, where commercially available primary airway smooth muscle (ASM), epithelial cells, and fibroblasts derived from human lungs were treated with 100 ng/mL of interleukin 13 (IL13), a cytokine known to be upregulated in asthma, for 6 hours and run on an Affymetrix Hugene FL microarray [21]. This study identified that treatment with IL13 caused dysregulation of a number of asthma-related genes. Differing effects were observed in different cell types of the airway, promoting the idea that each cell type plays its own role in asthma [21]. Once the ball started rolling, the asthma gene-expression microarray field quickly expanded from looking at single treatments on pure isolated cells in culture to the effects of more complex interactions including genes expressed during viral infection and direct comparisons of the gene expression in asthmatic and nonasthmatic tissue [22]. Although there have been many murine gene-expression microarray studies analyzing models of asthma, we have focused on the human studies in this review.

6.1. Airway Smooth Muscle Cells. The ASM plays a key role in the normal constriction and relaxation of the bronchial airway. In asthma the role of the ASM becomes exaggerated resulting in excessive airway narrowing in response to nonantigenic stimuli, termed AHR. A number of factors have been implicated in promoting AHR including airway remodeling and inflammation. The majority of the ASM gene-expression microarray studies to date have focused on the latter parameter and most focusing on the effect of IL13 [21, 23–25] but a small number have been conducted on remodeling [26]. In the search for an inflammatory mediator for asthma, IL-13 was found to play a critical role in murine asthma models [27]. During this time microarrays were just starting to be used in asthma research and many researchers took advantage of this new screening technology to help identify genes stimulated by this inflammatory cytokine. As already discussed the first of these studies was conducted by Lee et al., who identified a number of genes which were expressed specifically by ASM after treatment with 100 ng/mL of IL13 for 6 hours [21]. The next major study was conducted by Jarai et al. in 2004, who again looked at the effect IL13 had on ASM cells and two additional treatments, interleukin-1β (IL1β) and transforming growth factor-β (TGFβ) selected to identify if different inflammatory conditions cause ASM cells to distinct phenotype changes. Jarai et al. conducted this study using the updated Affymetrix GeneChip 95A array and stimulated ASM cells separately with 10 ng/mL of each treatment for 4 and 24 hours [23]. Although these authors conducted this study using cells derived from only two patients, a large range of genes induced by these three stimuli were identified [23]. IL1β stimulation confirmed the induction of a number of cytokines found in the literature to be previously upregulated; also a number of novel genes were identified including growth-related oncogene α, β, and γ, macrophage inflammatory protein 3α (MIP-3α), epithelial neutrophil activating peptide 78 (ENA-78), granulocyte-colony stimulating factor (G-CSF), and interleukin-1 receptor antagonist (ILRA) [23]. The main effect of IL13 stimulation on ASM was the induction of the expression of eotaxin, a strong chemoattractant for Th2-like T lymphocytes basophils and eosinophils which are found

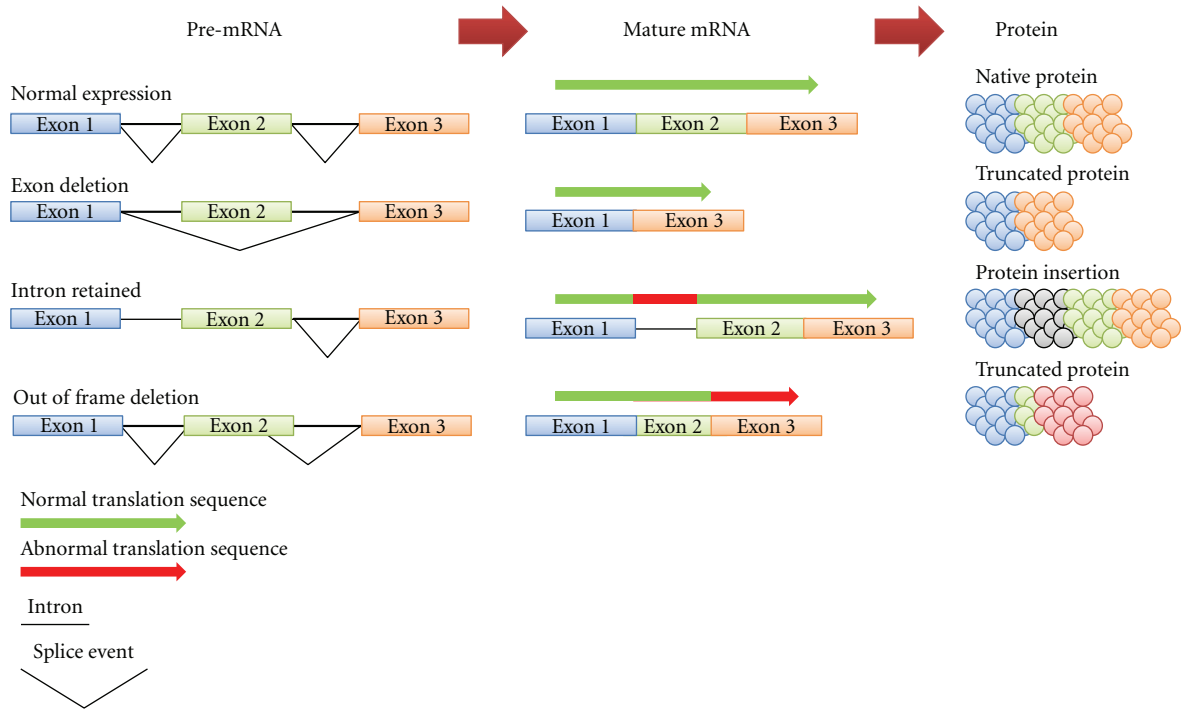

Figure 3: Transcripts from a single gene can undergo different splicing events. When mRNA is initially transcribed (known as pre-mRNA), it retains introns (thick black line), large segments of noncoding mRNA which separate exons, the coding regions. Through a process known as splicing, the introns are then removed and exons are ligated together to produce mature mRNA. Splicing also has the ability to remove exons or even retain introns resulting in the formation of different mature mRNA transcripts for the same gene (referred to as alternative splicing). Different mature mRNA transcripts encode for different proteins which may have alternative functions.

in tissues undergoing allergic reactions [28]. A note made by the authors was that this gene was not previously picked up by Lee et al. in their gene-expression microarray study and this disparity was thought to be due to differences in the concentration of IL13 and the sources of the ASM cells. TGFβ altered the expression of a number of structural and extracellular matrix proteins and also increased expression of IGF-binding protein 2, which had previously been indicated to mediate the growth response of TGFβ on ASM cells [23, 29].

High serum immunoglobulin E (IgE) levels have long been associated with allergic asthma [30]. In 2000 an association study identified a naturally occurring isoform of IL13 (IL13R130Q) to be associated with elevated serum IgE levels [27]. In an attempt to identify the role of IL13 and its isotypes in the pathogenesis of allergic asthma, Syed et al. looked at the effect of IL13 and IL13R130Q on ASM using an expression microarray containing 8159 human gene cDNA clones from Research Genetics (IMAGE consortium, Huntsville, AL), Incyte Genomics (Santa Clara, CA) [24]. No differences were detected between the genes induced by the two isoforms of IL13; however two genes, vascular cell adhesion protein 1 (VCAM1) and IL13 receptor alpha 2 protein chain (IL-13Rα2), were identified as being stimulated at both the mRNA and protein level [24]. VCAM1 had previously been implicated as a key player in the inflammation process; therefore the microarray further validated the role of IL13 in asthma [24]. IL13 induction of IL-13Rα2 was predicted by the author and that newly synthesized IL-13Rα2 may act as a decoy receptor therefore creating a self-regulating feedback loop preventing overstimulation of ASM cells, which had been previously confirmed in murine models [31].

6.2. Airway Epithelial Cells. The airway epithelium lies on the outermost layer of the bronchus and hence is positioned to directly respond to environmental irritants such as pollutants and viruses which are associated with asthmatic exacerbations. Previous studies have shown that asthma epithelium has alternations in both its structure and gene expression [32, 33]. The majority of gene-expression microarray studies focusing on the structural cells of the airway have focused on the epithelium [34–42]. Following the initial studies conducted by Lee et al., Yuyama et al. looked at the effect of Th-2 cytokines on human bronchial epithelial cells (n = 3) by treating them with 10 ng/mL of interleukin-4 (IL-4) and 50 ng/mL of IL13 separately for 24 hours before running the resulting cDNA on a Affymetrix Hugene FL microarray [34]. This study identified 2 major genes—squamous cell carcinoma antigen 1 (SCCA1) and squamous cell carcinoma antigen 2 (SCCA2) which were both increased by ~20 fold in both stimulations compared to untreated cells. SCCA1 and SCCA2 expression was found to be under the control of signal transducer and activator of transcription 4 (STAT4), a transcription factor previously found to be upregulated in epithelial cells derived from severe asthmatics [34]. Furthermore SCCA1 was found to be upregulated

in the serum of asthmatic patients especially during an asthma exacerbation [34]. Recently both SCCA1 and SCCA2 have been proposed as a method for testing for bronchial asthma attacks through the use of serum samples or mRNA expression [43].

In 2003 Kong et al. looked at early gene expression during a respiratory syncytial virus (RSV) infection of A549 epithelial cells using an Affymetrix Hugene FL microarray [35]. They found that two pathways, signal transducer and activator of transcription 1α and 3 (STAT-1α and STAT-3), were upregulated by RSV-key genes required for successful infection [44]. Subsequently the microarray identified 53 genes which had a change in expression due to RSV infection, consistent with changes in gene expression reported in previous studies [44].

Taking a different approach, Chu et al. looked at the effect of mechanical stress on gene expression in epithelial cells [36]. During bronchial constriction the epithelial layer experiences compressive mechanical stress [45], which in previous studies have been shown to stimulate the expression of transforming growth factor-β 2 (TGFB2) and endothelin (ET) facilitating fibrosis of the airway wall, a feature of asthmatic airways [46]. To identify further genes affected by mechanical stress, Chu et al. placed epithelial cells under mechanical stress for 8 hours and ran the samples against a set of pooled controls on Affymetrix Human 133A DNA microarrays [36]. Chu et al. identified a number of plasminogen-related genes (urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1), and tissue plasminogen activator (tPA)) which were upregulated on the microarray and confirmed both by qPCR and at the protein level [36]. Activation of the plasminogen pathways was shown to activate MMP-9, a protein associated with airway remodeling [47]. These results support the growing body of evidence that noninflammatory stimuli can contribute to the overall asthma phenotype [36].

In recent years two groups have conducted large screening for genes differentially expressed in asthma epithelium [41, 42]. The first of these two studies was conducted in 2007 by Woodruff et al. who compared the gene expression of 42 asthmatics and 44 nonasthmatics (28 healthy subjects and 16 smokers) and also the effect of corticosteroids on asthmatic patients using an Affymetrix Human 133A 2 plus microarray. The authors identified 22 genes which were found to be differentially expressed between asthma and healthy controls including periostin and serpinB2 and were verified by qPCR and at the protein level. The use of corticosteroids in asthmatic patients was shown to affect 30 genes compared to a placebo; 5 were verified by qPCR, including FK506 binding protein 51 (FKBP51), which had previously been identified to modulate glucocorticoid receptor activity [48].

The second study conducted in 2010 by Kicic et al. looked at the expression of children with asthma ($n = 36$), healthy atopic ($n = 23$) and healthy nonatopic controls ($n = 53$) using Affymetrix Human 133A DNA microarrays [42]. The aim of the study was to identify extracellular matrix (ECM) protein differentially expressed in asthma. They identified a single ECM gene fibronectin (FN) which was significantly lower in asthmatics, verified by qPCR and ELISA [42]. The authors then silenced FN expression in nonasthmatic epithelial cells and this was found to inhibit wound repair, while in the reverse situation the addition of FN to asthmatic epithelial cells restored wound repair within these cells [42]. Based on these results FN was identified as an ECM protein which may contribute to the abnormal epithelial repair seen in asthmatics.

6.3. Fibroblasts. Currently no further asthma-related gene-expression microarrays' studies have been conducted on human lung fibroblasts following the initial study conducted by Lee et al. [49]; however a number of arrays have been conducted on lung fibroblasts from murine models which have been reviewed elsewhere [50].

6.4. Mast Cells. Mast cells play an important role in asthma and other allergic disorders. Activation of mast cells by stimulatory factors, such as antigens or IgE, induces the production and/or release of cytokines and inflammatory mediators such as histamine. The use of gene-expression microarrays for human mast cell studies has been limited because of the difficulty of isolating this cell type [51–53]. The initial gene-expression microarray studies conducted on mast cells aimed to identify genes which were specifically expressed by mast cells. Nakajima et al. compared the expression of peripheral blood-derived mast cells, eosinophils, neutrophils, and mononuclear cells on an Affymetrix GeneChip 95A array [51]. They identified 140 genes which were mast cell specific and major basic protein (MBP) which were thought to be eosinophil specific. Furthermore MBP protein expression was verified by flow cytometry and confocal laser scanning microscopy. MBP is thought to be physiologically important in asthma as it has previously been found to be deposited in the damaged epithelium of asthmatic patients [54, 55].

6.5. Tissue. It has long been recognized that smooth muscle cells expanded in culture lose their contractile function and receptors with subsequent passaging [56]. The loss of function has also been noted in other cell types of the human airways. Therefore, the question has been raised as to whether gene-expression microarray studies on cultured cells give a true representation of physiological conditions. Tissue-based studies therefore provide a glimpse of the genes expressed under true physiological conditions, but because this source of mRNA is a mixture of cell types, it is impossible to differentiate which transcripts are being expressed by which cell type. One of the few tissue microarray expression studies, and the first 3′ microarray study to directly compare human asthmatic and nonasthmatic tissue, was conducted by Laprise et al. looking at the expression profile of biopsies before and after inhaled corticosteroid therapy [22]. Using an Affymetrix GeneChip 95A, Laprise et al. identified 74 genes which were differentially expressed between asthmatics and nonasthmatics, with a majority of these genes having already been identified as asthma related. However a number of novel genes were also identified including arachidonate 15-lipoxygenase (ALOX15), cathepsin C (CTSC), and chemokine (C-X3-C motif) receptor 1 (CX3CR1) [22].

Table 2: Asthma-related phenotypes that result from aberrant expression of splice variants.

Symbol	Gene Name	Phenotypes	Description	Reference
NPSR1	neuropeptide S receptor 1	IgE levels and Asthma	Isoform B over expressed in asthmatic ASM cells	[15]
IL-4	interleukin-4	Asthma	Alternatively spliced variants of IL-4 mRNA are differently expressed in patients with bronchial asthma	[58, 59]
COX-1	cytochrome c oxidase assembly homolog (yeast)	Asthma	Alternatively spliced variants of COX-1 mRNA are differently expressed in patients with bronchial asthma	[60]

Comparing asthmatic subjects before and after inhaled corticosteroid therapy identified 128 genes which had altered expression in the presence of the therapy. However 70% of the genes which were upregulated in asthma remained unchanged after corticosteroid therapy [22]. It was predicted by the author that a subset of these genes may be responsible for asthma chronicity [22].

However 3′ arrays only provide the user with the overall level of gene expression without measuring the degree of contribution of different splice variants of the genes being interrogated to the total gene expression. This is problematic, as a number of key asthma-related genes including NPSR1, IL-4, cytochrome c oxidase assembly homolog (yeast) (COX-11), and ADAM33 have been found to have dysregulation of alternative splicing patterns and/or differential expression of specific splice variants [15, 57–60].

7. Alternative Splicing

The human genome contains ∼30,000 genes; however it has been predicted that there are over 100,000 proteins expressed in the body. These predictions are in contrast to the previously well-accepted "one gene-one enzyme" theory proposed in 1941, where it was believed that each gene encoded only one protein [61]. Recently, alternative splicing has been identified as the process through which this apparent gene deficiency in the human genome is explained. When mRNA is initially transcribed (known as pre-mRNA), it retains introns, large segments of noncoding mRNA which separate exons, the coding regions (Figure 3). Through a process known as splicing, the introns are then removed and exons are ligated together to produce mature mRNA sequences. Splicing also has the ability to remove exons or even retain introns resulting in the formation of different mature mRNA transcripts for the same gene (referred to as alternative splicing).

It is now predicted that over 95% of all multiexon genes in the human genome undergo some degree of alternative splicing [62]. Depending on what sections of RNA are removed or retained, alternative splicing can have major effects on the functionality of the resultant proteins (Figure 3). Therefore, it is not surprising that a number of genetic diseases including asthma have been linked with mutation and changes with cis- (e.g., DNA sequence related) and trans- (e.g., transcription factors) acting factors which lead to aberrant splicing and irregular protein production (Table 2).

The function of disease causing splice variants is still poorly understood; however, based on previous findings (Table 2), studying splice variants may provide an untapped resource which could hold some answers for their role in asthma.

8. The Future of Gene-Expression Microarrays: Affymetrix HuExon 1.0 ST

Recent advances in gene-expression microarray design have produced the Affymetrix HuExon 1.0 ST; this platform allows for the evaluation of not only gene expression but also exon level expression and identification of unknown splicing events. The Affymetrix HuExon 1.0 ST contains 65 million probes, 8 times the number of the probes present in the latest release of the Affymetrix U133 Plus2 array. The main differences between exon arrays and 3′ arrays come from the number and binding sites of the oligonucleotide probes, labeling methods and differing methods for identifying background noise levels.

3′ arrays simply use 11–20 probes for each gene which bind to the 3′ end, while Exon arrays use ∼40 probes evenly spaced along all exons of a given transcript. The advantage of this method is that Exon arrays can detect all isoforms of a gene transcript and evaluate the level of expression for each splice variant, while 3′ arrays lack this ability as their probes are only positioned towards the 3′ end of an mRNA transcript.

Another key difference is the generation of the sample mRNA during the initial cDNA step; Exon arrays use random probes (containing 6 random nucleotides) attached to a T7 promoter whereas oligo(dT)s attached to a T7 promoter are used as the primers for the 3′ arrays. By using random probes which bind anywhere on the mRNA transcript (not restricted to the poly-A tail), Exon arrays have overcome the problem of identifying non-polyadenylated transcripts by covering the entire gene transcript, rather than having a 3′ bias. Replacing the function of the MM probe control used by 3′ arrays, Exon arrays use a specially designed set of probes which should not bind any mRNA to detect background binding. Exon arrays represent many improvements when compared to their predecessors and, as the search for disease causing candidates moves forward, it will only be a matter of time

before these arrays receive much greater attention in the scientific community.

8.1. Affymetrix HuGene 1.0 ST Microarray. The Gene 1.0 ST Array was designed based on the Exon 1.0 ST Array (and therefore uses the same sample preparation and labeling methods). The key difference is that it contains only a subset of the probes, focusing on well-annotated content. Similar to 3′ arrays, the Affymetrix Gene 1.0 ST also provides a platform for measuring genomewide gene expression of a sample at a single point in time. However, by using probes which are evenly spaced along all exons, the Affymetrix Gene 1.0 ST array is able to give a true representation of gene expression (Figure 2). Gene 1.0 ST arrays also have a limited ability to identify alternatively spliced gene products; however the low number of probes per exon means that false positive events occur more commonly than with the superior Human Exon 1.0 ST Array.

8.2. The Influence of Exon Array Asthma Research. At the time of writing this review the use of exon arrays in reported human asthma-related projects was limited to a single study conducted by Plager et al. 2010 [63]. This study aimed to identify genes related to asthmatic chronic rhinosinusitis with nasal polyps (aCRSwNP) and eosinophilic epithelial inflammation, through the use of an Affymetrix HuExon 1.0 ST. Although alternative splicing was not looked at in this study, Plager et al. identified a number of chemokines and chemoattractants including eotaxin-1 (associated with inflammation and upregulated by IL13 [23, 28]), -2, and -3 which were associated with aCRSwNP.

9. MicroRNA

MicroRNAs (miRNA) are short (22 nucleotide) segments of RNA which bind to complementary sequences on target mRNA, thereby facilitating mRNA degradation and thus repressing gene expression at the transcriptional level. miRNAs can be transcribed from their own genes or exist within intronic regions of mRNA. miRNAs are incorporated into miRNA-argonaute complexes which facilitate their ability to degrade/inhibit mRNA transcripts (reviewed in [64]). The human genome is believed to encode over 1000 miRNAs [65], and these miRNAs are predicted to bind to over 60% of all mRNA transcripts in the human genome [66]. The dysregulation of miRNAs has been identified in a number of human diseases including asthma [67].

10. MiRNA Microarray: How It Works

There are a large range of miRNA microarrays currently being offered by a number of companies (Table 3); however, as yet, the methodology used to analyze these arrays has not been standardized. In this review we will discuss the methods used by the *mir*Vana miRNA bioarrays V2 (Ambion) which were used by Kuhn et al. [67], currently the only miRNA array conducted on human ASM cells (Figure 4). The *mir*Vana miRNA bioarray, like a number of other miRNA microarrays on the market, relies on the addition of a poly (A) tail containing modified adenosine nucleotides to all remaining RNA (after miRNA purification) which allows for the specific binding of fluorescent dyes, prior to being hybridized to the array.

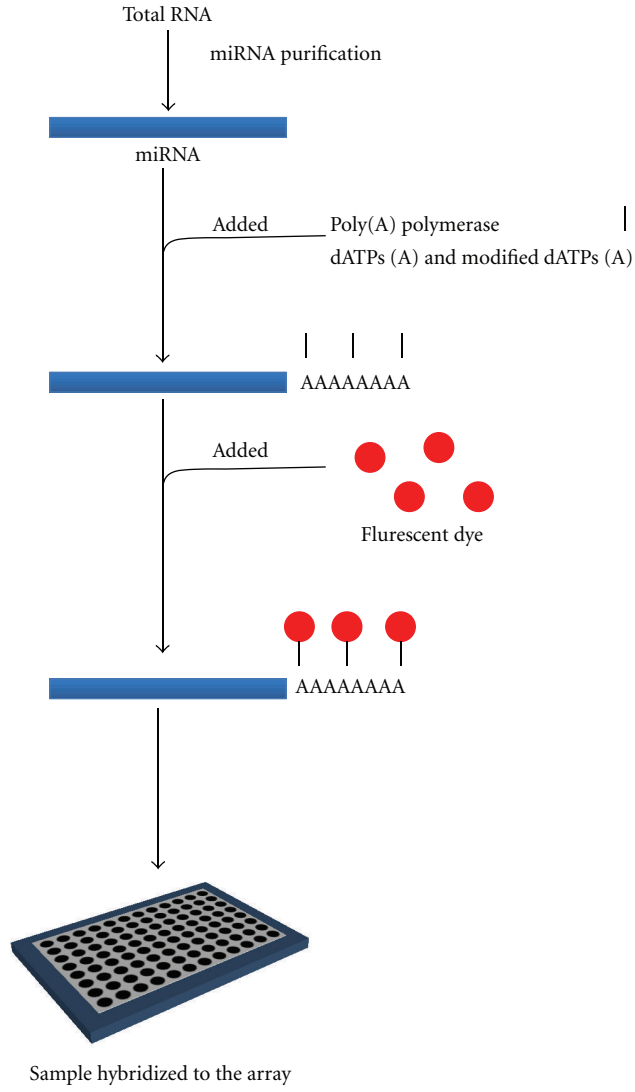

Figure 4: Overview of *mir*Vana miRNA bioarray methodology. Total RNA is extracted from tissue or cells and miRNA purified. Poly(A) polymerase is then added in the presence of modified dATPs and normal dATP. A poly(A) tail containing the modified dATPs is then added to all RNAs present in the sample. Fluorescent dye is added which binds to the poly(A) tail and the sample is hybridized to the array.

miRNA asthma research is still in its infancy, with the initial report of a human asthma miRNA array study being made by Kuhn et al. in 2010 [67]. These researchers looked at the effect of IL-1β, TNF-α, and IFN-γ on miRNA expression in ASM cells using *mir*Vana miRNA bioarrays V2 (Ambion). miR-25, miR-140*, miR-188, and miR-320 were found to be significantly downregulated, and these data were verified by both microarray and quantitative PCR. Furthermore miR-25 had previously been identified

Table 3: List of a number companies currently providing miRNA microarray technology.

Company	Microarray	Link
Ambion	*mir*Vana miRNA bioarrays V2	http://www.ambion.com/
Agilent Technologies	Human miRNA Microarray Release 16.0	http://www.genomics.agilent.com/
Affymetrix	GeneChip miRNA 2.0 Array	http://www.affymetrix.com/
Exiqon	miRCURY LNA microRNA Array	http://www.exiqon.com/
Invitrogen	NCode Human miRNA Microarray V3	http://products.invitrogen.com/
LC Sciences	V17.0 Human microRNA Microarray	http://www.lcsciences.com/
Miltenyi Biotec	miRXplore Microarray Kits	http://www.miltenyibiotec.com/
System biosciences	miRNome MicroRNA Profilers	http://www.systembio.com/

Table 4: The GEO accession number for microarray studies conducted on asthma.

Year	Title	Array	GEO accession number	Reference
	Smooth muscle cells			
2001	Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types	Affymetrix Hugene FL	n/a	[21]
2004	Effects of interleukin-1 [beta], interleukin-13, and transforming growth factor-[beta] on gene expression in human airway smooth muscle using gene microarrays	Affymetrix GeneChip 95A	n/a	[23]
2005	The effect of IL13 and IL13R130Q, a naturally occurring IL13 polymorphism, on the gene expression of human airway smooth muscle cells	8159 human gene cDNA clones from Research Genetics (IMAGE consortium, Huntsville, AL), Incyte Genomics	n/a	[24]
2007	1α,25-Dihydroxy-vitamin D_3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes	Human Genome U133 Plus 2.0 GeneChip arrays	GSE5145	[26]
2009	Glucocorticoid- and protein kinase A-dependent transcriptome regulation in airway smooth muscle	Affymetrix Human U133A DNA microarrays	GSE13168	[25]
2010	MicroRNA expression in human airway smooth muscle cells: role of miR-25 in regulation of airway smooth muscle phenotype	*mir*Vana miRNA bioarrays V2 (Ambion)	GSE16587 GSE16586	[67]
	Epithelial cells			
2002	Analysis of novel disease-related genes in bronchial asthma	Affymetrix Hugene FL	n/a	[34]
2003	Respiratory syncytial virus infection activates STAT signaling in human epithelial cells	Affymetrix Hugene FL	n/a	[35]
2006	Induction of the plasminogen activator system by mechanical stimulation of human bronchial epithelial cells	Affymetrix Human 133A DNA microarrays	n/a	[36]
2007	IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production	UCSF 9Hs Human 23K v.2 Oligo Array	GSE4804	[37]
2007	Genomewide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids	Human Genome U133 Plus 2.0 GeneChip arrays	GSE4302	[41]
2009	Airway epithelial cells regulate the functional phenotype of locally differentiating dendritic cells: implications for the pathogenesis of infectious and allergic airway disease	Human Genome U133 Plus 2.0 GeneChip arrays	GSE12773	[38]
2009	T-helper type 2-driven inflammation defines major subphenotypes of asthma	Human Genome U133 Plus 2.0 GeneChip arrays	GSE4302	[39]
2010	Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma	Human Genome Focus GeneChip Array Human Genome U133 Plus 2.0 GeneChip arrays	GSE13396	[40]
2010	Transglutaminase 2, a novel regulator of eicosanoid production in asthma revealed by genomewide expression profiling of distinct asthma phenotypes	Affymetrix Human U133A DNA microarrays Human Genome U133 Plus 2.0 GeneChip arrays	GSE13785	[83]
2010	Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium	Affymetrix Human 133A DNA microarrays	GSE18965	[42]

Table 4: Continued.

Year	Title	Array	GEO accession number	Reference
	Mast Cells			
2001	Gene expression screening of human mast cells and eosinophils using high-density oligonucleotide probe arrays: abundant expression of major basic protein in mast cells	Affymetrix GeneChip 95A	n/a	[51]
2005	Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts	Affymetrix Genechip Human Genome U133	n/a	[52]
2009	Human mast cells synthesize and release angiogenin, a member of the ribonuclease A (RNase A) superfamily	NIAID (human sequence chip series "sa") and consist of 13,971 oligonucleotides, synthesized by Qiagen Operon Inc. (Valencia, CA, USA)	n/a	[53]
	Tissue			
2004	Functional classes of bronchial mucosa genes that are differentially expressed in asthma	Affymetrix GeneChip 95A	GSE15823	[22]
2010	Gene transcription changes in asthmatic chronic rhinosinusitis with nasal polyps and comparison to those in atopic dermatitis	Affymetrix HuExon 1.0 ST	GSE5667	[63]

GEO: NCBI Gene Expression Omnibus.
n/a: microarray data not submitted to a database or not stated in paper.

to regulate Krüppel-like factor 4 (KLF4), a mediator of proinflammatory signaling in macrophages [68]. Kuhn et al. confirmed that the downregulation of miR-25 allowed for the upregulation of KLF4 [67].

The role of miRNAs in asthma is still under investigation; however by identifying the role of certain miRNAs by up/downregulation of its expression and measuring its effects on overall gene expression (microarray) or using prediction software to predict genes which the miRNA may bind to and following this up by Real time PCR, researchers are slowly identifying the function of particular miRNA. In the future, miRNA may provide a source of novel treatments and therapies.

11. Challenges for Gene-Expression Microarray Projects

Despite numerous advantages, the use of microarrays still has many limitations, mainly relating to the experimental design, sample variance, and the statistics used to analyze the accumulated data. The challenges of microarray statistics in complex diseases have previously been extensively reviewed [69] and will not be discussed here.

11.1. Experimental Design. One of the main limitations of microarray studies today is still the cost; however this is slowly decreasing. In the past, cost was a major burden, often limiting the number of patients/samples analyzed in previous microarray studies; Jarai et al. and Yuyama et al. looked at the effect of Th-2 cytokines on human bronchial epithelial cells ($n = 3$) and primary ASM cells ($n = 2$) [23, 34]. Another contributing factor to the lack of patients analyzed is the concept of replicates versus treatments; when dealing with a limited number of arrays a decision one must make is whether to sacrifice the number of replicates to allow for an increase in the number of treatments studied, or vice versa. Traditionally, when dealing with different treatments of the same cell type which encompass the majority of asthma-related microarray studies, studies were designed to increase the number of treatments. The low patient/sample per treatments number was then overcome by conducting followup experiments such as Quantitative Real-time PCR and/or ELISA with a greater patient pool on single candidate genes [23, 34]. However, this still leaves an extensive list of genes to followup which is usually impractical with these alternative methods.

11.2. Sample Variance. Factors such as patient-to-patient variation and the heterogeneity of the asthma phenotype can make microarray data unreliable and hard to replicate. Therefore, it is important that the right type and number of patients are selected for each study, hence reducing the variation within the samples. A problem that many asthma studies face is obtaining samples with similar asthma diagnoses. Using patients who have the same level of severity of asthma and diagnosis using the same defined method can help reduce this variation. Also, it is important to ensure that the patients analyzed have no other underlying airway disease or other comorbidity. Accessing samples from pure patient populations can be challenging; however it can greatly decrease the number of false positives within the resulting microarray dataset.

12. Accessing Previous Microarray Study Data

In 2002, the Nature family of journals adopted the minimum information about microarray experiments (MIAMEs) standard (developed by the Microarray Gene Expression

Data Society (MGED) [70]), making it mandatory that all microarray data (including annotations) used in publishable research must be deposited into an acceptable public repository (NCBI Gene Expression Omnibus (GEO) [71], ArrayExpress [72], or Center Information Biology Gene Expression Database (CIBEX) [73], prior to the submission of a manuscript [74]. There are six key elements within the MIAME guidelines which authors must provide:

(1) raw data for each microarray (e.g., cel files),

(2) the processed normalized data,

(3) annotation of the samples used to conduct the microarrays (treatment, cell types, etc.),

(4) the experimental design,

(5) annotation of the array itself (coordinates of probes and their sequences),

(6) methods of normalizing the data to obtain the processed data.

Over the years, many other journals have also adopted the MIAME standard, turning GEO (http://www.ncbi.nlm.nih.gov/geo/) into a free microarray database containing ~20,000 microarray studies to date [75]. Because this information is freely available, researchers now have the opportunity to design specific questions and search for a previous microarray project to help narrow down the list of candidate genes involved with their function of interest; the GEO accession numbers for all microarrays' studies discussed in this review are highlighted in Table 4. However, not all the scientific community believe that the MIAME guidelines are beneficial. A number of critics have expressed the view that forcing groups to disclose their microarray data upon publishing has led to many to groups simply not publishing their findings. Whether this restriction is resulting in a biased reporting of the application of microarrays in research is yet to be determined. Another issue is the inability of researchers to repeat the analysis of published expression microarrays in MIAME abiding journals. This problem was recently discussed in a paper by Ioannidis et al. [76]. In this study Ioannidis and colleagues attempted to replicate the data analyses in 18 articles on microarray-based gene expression published in Nature Genetics in 2005-2006. Of the 18 articles ten could not be reproduced [76]. The main reasons for this were data unavailability (even though these articles were published under the MIAME guidelines) and incomplete data annotation not abiding by the MIAME guidelines [76]. Based on this study and a number of reviews it is clearly evident that MIAME provides a method of allowing researchers to share and scrutinise microarray based data by providing the necessary information; however it is only as effective as the enforcement that journals which abide by these guidelines impose on researchers to make sure that they follow them correctly [76, 77].

13. Conclusion

Microarrays have significantly increased our understanding of the genes and cell types involved with asthma. Although the use of microarrays in asthma research is still at an early stage, it has helped confirm the results of previous studies and has identified a number of novel genes which warrant further, investigation. As the price of microarrays decreases and the technology advances further the use of microarrays in asthma-related research will expand and may provide exciting new insights into the genetic regulation of this complex pathological process.

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Review Article

Role of the Arylhydrocarbon Receptor (AhR) in the Pathology of Asthma and COPD

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The dioxins and dioxin-like compounds in cigarette smoke and environmental pollutants modulate immunological responses. These environmental toxicants are known to cause lung cancer but have also recently been implicated in allergic and inflammatory diseases such as bronchitis, asthma, and chronic obstructive pulmonary disease (COPD). In a novel pathway of this response, the activation of a nuclear receptor, arylhydrocarbon receptor (AhR), mediates the effects of these toxins through the arachidonic acid cascade, cell differentiation, cell-cell adhesion interactions, cytokine expression, and mucin production that are implicated in the pathogenesis and exacerbation of asthma/COPD. We have previously reported that human bronchial epithelial cells express AhR, and AhR activation induces mucin production through reactive oxygen species. This review discusses the role of AhR in asthma and COPD, focusing in particular on inflammatory and resident cells in the lung. We describe the important impact that AhR activation may have on the inflammation phase in the pathology of asthma and COPD. In addition, crosstalk of AhR signaling with other ligand-activated transcription factors such as peroxisome proliferator-activated receptors (PPARs) has been well documented.

1. Introduction

Both allergic asthma and COPD are defined as airway inflammatory diseases; however, the inflammatory mechanism is different for each disease. Nocuous agents such as PCBs, B[a]P, and dioxin-like compounds in cigarette smoke and environmental pollutants have the potential to induce inflammation or exacerbate chronic bronchitis, asthma, COPD, and lung cancer [1–4]. In addition to airway epithelial cells, many inflammatory cells, including Th2 cells, eosinophils, and basophils, play a major pathophysiological role in asthma and COPD [5–8]. Cigarette smoke and environmental pollutants activate these inflammatory cells, and they contribute to the activation of growth factors and cytokines. For example, exposure to some types of noxious agents increases the rate of TGF-α, TGF-β, IL-1β, IL-6, IL-8, and IFN-γ gene expression [9–12]. While the molecular signaling mechanism for this transcriptional modulation of cytokines remains to be determined, it has been recently recognized that these effects are mainly mediated through the binding of noxious agents to the AhR. All major human cell types express AhR, including pulmonary tissue [13, 14]. The liver, adipose tissue, and skin are the major storage sites of AhR ligands in humans [15]. These AhR ligands are also concentrated in bronchial epithelial cells, suggesting that the respiratory system is sensitive to AhR ligands [16].

The AhR is a ligand-activated transcription factor, and after ligation of dioxins to the AhR, the receptor translocates from the cytosol to the nucleus, where it heterodimerizes with the ARNT. It then binds to a DRE, an enhancer sequence of several drug-metabolizing enzymes, such as CYP1A1 [17]. AhR-induced CYP1A1 activation is important for detoxication. CYPs convert B[a]P and dioxin-like compounds into physiologic metabolites that exert effects on cell growth, differentiation, and migration. A number of researchers have demonstrated the molecular aspects of the AhR pathway by using selective agonists such as TCDD or B[a]P among PAHs.

In this review article, we summarize current findings regarding the functional role of AhR molecules in airway inflammation and focus on bronchial epithelial cells, fibroblasts, granulocytes, and lymphocytes. Understanding the effects of AhR on these cells would be a breakthrough in our understanding of the pathology and treatment of asthma and COPD.

2. Airway Inflammatory Effect through AhR Activation in Asthma and COPD

2.1. Airway Epithelial Cells. Airway epithelial cells are able to modify allergic airway inflammation by virtue of their ability to produce a variety of inflammatory mediators [18, 19]. One such mediator is the moderate bronchial mucin-containing mucus, which normally protects the airway from exogenous substances. Hypermucosis in the airway, however, is associated with several respiratory diseases, including asthma and COPD. Mucus hypersecretion in the airway increases coughing and expectoration of sputum. Clara cells in the airway can secrete a wide variety of glycoproteins, such as mucins and SP-D, and are very sensitive to AhR stimulation [20, 21]. Wong et al. recently have reported TCDD, an AhR agonist, increased expression of inflammatory cytokines, MUC5AC, and MMPs via AhR signaling in a Clara-cell-derived cell line [21]. Mucus production is typically mediated by cytokine or lipid mediator release, or an increase of ROS [22–24]. Studies using AhR agonists and inhibitors have demonstrated that AhR activation induces the production of cytokines such as TGF-α, TNF-α, and MMP through receptors in human hematocytes and epithelial cells [21, 25–27]. Wong et al. also reported an increase of COX-2 and IL-1β mRNA expression in response to AhR activation [21]. The production of prostanoids such as PGE_2, which is derived from COX-2, can activate mucin production in the airway [22]. Although prostaglandins derived from COX-2 pathway activation may be responsible for AhR-induced mucin production in the bronchial epithelial cells, the mechanism of their action remains to be determined. Therefore, it is of paramount interest to investigate the mechanism by which AhR activation induces mucin production. In an earlier study, we reported findings similar to those by Wong et al. In our study, we found that AhR activation upregulates the expression of MUC5AC and mucin secretion in a NCI-H292 cell line that was derived from a bronchiolar Clara cell [14] (Figure 1). Moreover, we concurrently showed that AhR activation induced ROS generation, and the antioxidant agent NAC inhibited B[a]P-induced MUC5AC upregulation. Kopf and Walker also demonstrated that TCDD-induced AhR activation increased ROS levels in endothelial cells [29]. Another prostaglandin, PGD_2, is synthesized from arachidonic acid via the catalytic activities of COX in epithelial cells and mast cells. It is released into the airway following an antigen challenge during an acute allergic response [30]. PGD_2 induces chemotaxis of Th2 cells, eosinophils, and basophils as a consequence of the activation of its receptors [31]. This suggests that PGD_2 promotes inflammation in allergic asthma. Prostaglandins that are derived from COX-2 pathway activation and ROS that are induced by AhR activation are the major inflammatory mediators capable of inducing mucin production, inflammatory cell chemotaxis, or inflammatory cell activation. Therefore, increased levels of prostaglandins and ROS, either directly or through the formation of lipid peroxidation products, may enhance the inflammatory response in both asthma and COPD.

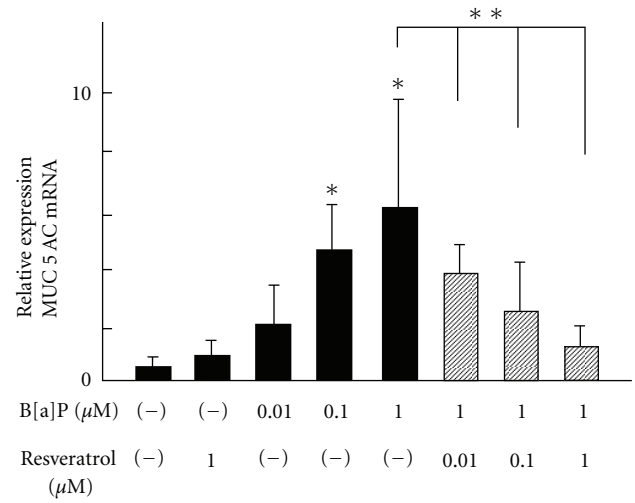

FIGURE 1: Effects of AhR agonist B[a]P on MUC5AC mRNA level in NCI-H_{292} after 12 h of incubation. MUC5AC was measured by real-time RT-PCR. B[a]P induced MUC5AC mRNA expression in dose-dependent manner. Pretreatment with AhR antagonist, resveratrol, inhibited AhR-induced MUC5AC upregulation. Data are expressed as means ± SD ($n = 6$). $^*P < 0.05$ versus control (medium alone). $^{**}P < 0.05$ versus B[a]P 1 μM.

Neutrophils isolated from peripheral blood and BAL fluid of asthmatic patients generate more ROS than cells from normal patients. Additionally, the production of ROS correlates with the degree of airway hyperresponsiveness [32, 33]. Neutrophils and macrophages are also known to migrate into the lungs of COPD patients [34, 35]. Indeed, the neutrophils that mediate ROS-induced injury to the airway epithelium are responsible for hyperresponsiveness in human peripheral airways, suggesting that neutrophils play an important role in the pathogenesis of asthma and COPD [36]. AhR-derived inflammatory mediators in airway epithelial cells, such as IL-8 and leukotriene B4, may have a chemotactic effect. We previously confirmed that normal human epidermal keratinocytes (NHEKs) enhanced IL-8 production through AhR activation [37] (Figure 2). Martinez et al. demonstrated that IL-8 gene expression was upregulated by TCDD in A549 cells from a bronchial epithelial cell line [38]. However, they could not detect IL-8 production at the protein level in airway epithelial cells. We were also unable to detect IL-8 production from AhR-activation in NCI-H292 cells using ELISA analysis (data not shown). Although it is not clear that AhR directly modulates NF-κB, the induction of a transcription factor for IL-8, tumor necrosis factor, or IL-1β by AhR activation might impact IL-8 production in airway epithelial cells [21, 25, 26, 39].

Cell-cell contact molecules in the airways create a barrier that plays an important role in the defense against bacteria.

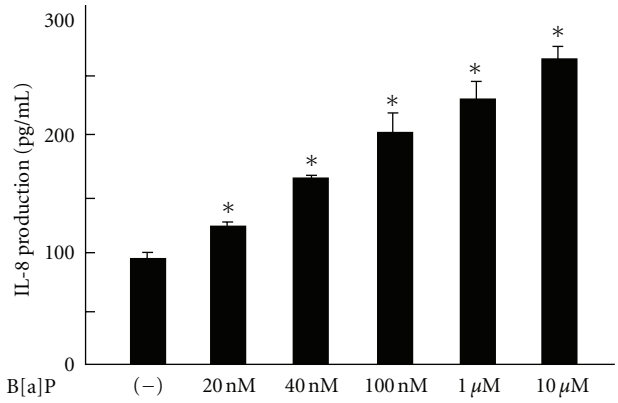

Figure 2: Normal human epidermal keratinocytes (NHEKs) were exposed to B[a]P at various concentrations for 24 h, and IL-8 production in the culture supernatant was measured. B[a]P induced IL-8 production in a dose-dependent manner. Data are expressed as means ± SD ($n = 3$). $^*P < 0.05$ versus control (medium alone).

Loss of expression of cell-cell contact molecules, such as E-cadherin, reduces the ability of epithelial cells to function as a barrier and may increase the allergic response and susceptibility to infection. Indeed, E-cadherin and α-catenin interacted with cytosolic domain of the cadherin expression are significantly lower in asthmatic than in nonasthmatic subjects [40]. AhR also regulates the expression of adhesion molecules and consequently controls cell-cell contact. Exposure to TCDD from a human breast cancer cell line downregulates E-cadherin expression [41]. Using rat liver epithelial cells, Dietrich et al. demonstrated that TCDD exposure inhibits the expression of γ-catenin, which links E-cadherin to actin filaments [42]. We hypothesize that several pathways may be involved in the production of inflammatory cytokines and mucus in asthma and COPD, as illustrated in Figure 3.

2.2. Fibroblast or Airway Smooth Muscle. Chronic asthmatic patients who are unresponsive to treatment experience progressive and irreversible changes in pulmonary function. These changes, known as "airway remodeling," are associated with structural alterations, such as subepithelial fibrosis, smooth muscle or goblet cell hyperplasia, and airway hyperresponsiveness [43]. In chronic asthma patients, fibrosis is due to increased deposition of extracellular matrix. Increases in airway smooth muscle mass are thought to be caused by faster proliferation, mitogenic, or inflammatory stimuli [44]. Some of the factors contributing to these effects are TGF, FGF, EGF, and PDGF. TGF-β is one of these contributors and is a major effector cytokine that can increase deposition by fibroblasts and airway smooth muscle hypertrophy. Guo et al. reported that levels of RNA for TGF-β2 and TGF-β2-related genes increased in AhR-knockout smooth muscle cells [45]. This suggests that AhR may repress the TGF-β- signaling pathway, resulting in an anti-inflammatory effect unlike in rodent lung cells. On the other hand, cigarette smoke, via AhR, can induce cyclooxygenase and PGE_2 in human lung fibroblasts [46]. PGE_2 significantly enhances cigarette smoke extract-treated neutrophil chemotaxis and adhesion to airway epithelial cells [47]. In fact, the concentration of PGE_2 in the sputum of COPD patients is correlated with the number of infiltrating neutrophils [47]. Neutrophil activation through AhR signaling plays a causal role in pathogenesis and exacerbation of COPD.

2.3. Granulocytes with Focus on Eosinophils. Eosinophils play an essential role in the pathology of asthma because they contribute to tissue injury, vascular leakage, mucus secretion, and tissue remodeling by releasing cytotoxic granule proteins, ROS, and lipid mediators [48]. Because eosinophils are the final effector cells in allergic inflammation, it is important to study the process by which nuclear receptors, such as AhR, activate eosinophils in order to understand the pathogenesis of allergic diseases. For example, PPARs are among the important ligand-activated transcription factors that regulate the expression of genes involved in many cellular functions, including differentiation, immune responses, and inflammation [49, 50]. The PPAR subfamily consists of 3 isotypes: PPARα, PPARβ/δ, and PPARγ, all of which have been identified in eosinophils. These nuclear receptors form heterodimers with retinoid X receptors, bind to a specific DNA sequence (PPRE), and activate target gene transcription. *In vivo* and *in vitro* evidence suggests that PPARα and PPARγ expression in granulocytes and dendritic cells plays a critical role as an inflammatory suppressive regulator in allergic diseases. Treatment with the PPARγ agonist, rosiglitazone, decreases the clinical severity of skin lesions in atopic dermatitis and airway inflammation in asthmatic patients [51, 52]. We previously demonstrated that the PPARγ agonist troglitazone reduced IL-5-stimulated eosinophil survival, eotaxin-directed eosinophil chemotaxis, and functional augmentation of eosinophil adhesion in a concentration-dependent manner. These changes occurred without reducing the quantitative expression of β2 integrins [53, 54] (Figure 4). It has been lately shown that PPARγ induction is suppressed during the activation of the AhR by TCDD [55]. In addition, Cho et al. demonstrated that CYP1B1 upregulation induced the inhibition of AhR expression in 10T1/2 cells derived from preadipocyte lines. Moreover, the reduced AhR expression was accompanied by an increase in PPARγ expression [56]. These results suggest that the AhR signal may repress migration, degranulation, and cellular adhesion of eosinophils. This may impair the antiallergic effects induced by PPARγ. We were able to confirm AhR expression in human eosinophils using RT-PCR (data not shown). Clarification of the interaction between AhR and PPARγ signals should broaden our understanding not only of the functional role of eosinophils but also of asthma regulation.

2.4. Lymphocytes. Allergic asthma is associated with disruption of the immune system, particularly an imbalance of Th1 and Th2 cells. It is well known that Th2 cells play a key role in the regulation of inflammatory reactions through the release of Th2 cytokines. AhR is known to exert an influence on allergic immunoregulation. In fact, Tauchi et al. reported that mice with constitutive AhR activation

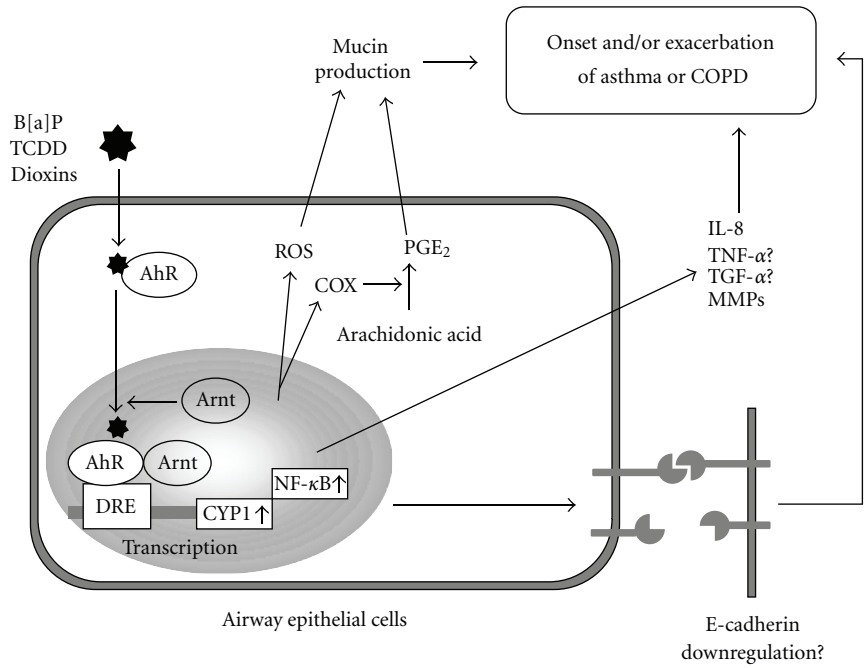

Figure 3: Schematic diagram of the proposed crosstalk AhR-signaling pathway and inflammatory effects in airway epithelial cells.

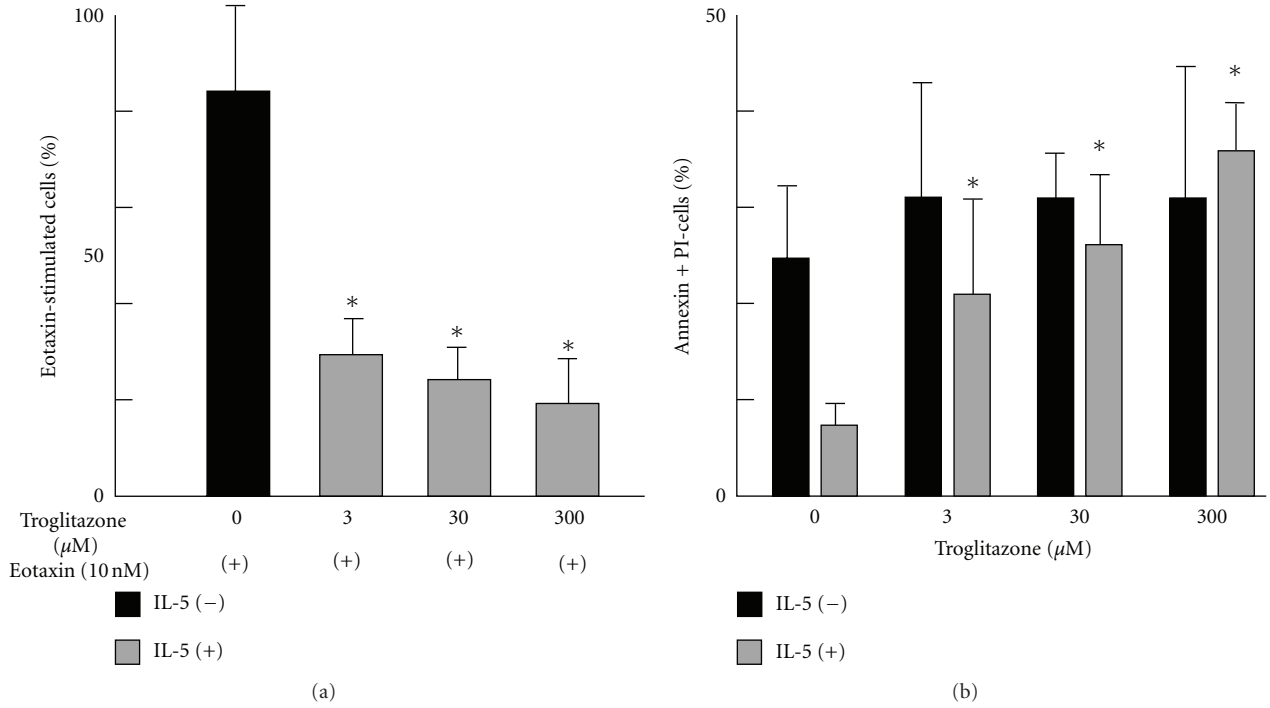

Figure 4: (a) Effect of PPARγ agonist troglitazone on eosinophil chemotaxis stimulated with eotaxin. Purified eosinophils were preincubated with increasing concentrations of troglitazone for 1 h. Migration assays were performed using Boyden chambers. Chemotactic response to eotaxin alone was considered to be 100%, and reactions to lower concentrations are presented relative to eotaxin alone. Data are expressed as mean ± SD. Troglitazone inhibited the eotaxin-directed eosinophil chemotaxis in a dose-dependent manner ($n = 4$). $^*P < 0.05$ versus eotaxin alone. (b) Effect of troglitazone on eosinophil survival determined by staining with Annexin V-FITC and propidium iodine. Eosinophils were incubated with and without troglitazone in the presence of 1 ng/mL IL-5 for 48 h. Eosinophils were treated with Annexin V to stain early-phase apoptotic cells and with propidium iodine (PI) to stain the late-phase cells. The bar graph shows a dose-dependent effect of troglitazone on IL-5-induced eosinophil survival ($n = 4$). Data are expressed as mean ± SD. $^*P < 0.05$ versus without troglitazone.

developed severe skin lesions that were similar to the lesions seen in atopic dermatitis. The lesions were accompanied by high serum levels of IgE and increased production of IL-4 and IL-5 from stimulated splenic lymphocytes [57]. In addition, AhR expression in splenic B cells was enhanced by the presence of lipopolysaccharide, which is known to exacerbate asthma and COPD [58]. PAH and TCDD increase IgE production in cocultures with purified B cells [59]. These results provide further evidence that AhR may play a complex role in the humoral immunological balance in airway allergic pathogenesis.

Th17 cells have been recently classified as a subtype of helper T cells that are characterized by the production of IL-17 [60]. AhR activation promotes the development of Th17 cells and results in increased pathology in animal models of multiple sclerosis [61]. Th17 cells found in the skin, gastrointestinal tract, and bronchial tubes are involved in inflammatory conditions, such as inflammatory bowel disease and asthma [62]. The IL-17 produced by Th17 cells is a potent activator of NF-κB, thereby, increasing the levels of inflammatory cytokines such as IL-8, IL-6, TNF-α, G-CSF, and GM-CSF [63]. Therefore, although Th17 plays a role in regulating neutrophil and macrophage inflammation, it is not known whether IL-17 induced by AhR activation contributes to the development of asthma or COPD. Clinically, IL-17 levels in BAL fluid, sputum, and peripheral blood from patients with allergic asthma are higher than those in healthy controls [64, 65]. A knockout mouse model of the IL-17 receptor showed reduced OVA-induced airway hyperresponsiveness and eosinophil infiltration. Additionally, the levels of IgE and Th2 cytokines in knockout mice were not as highly elevated as they were in wild-type mice [66]. Furthermore, stimulation with IL-17 increased the concentration of biologically active MMP-9 in mouse airways. IL-17 protein, as represented by neutrophilic inflammation, has been detected in COPD patients, but at a lower level than observed in asthma patients [67]. Human lymphocytes, however, may behave differently. For example, AhR agonists appear to favor IL-22 but not IL-17 production in humans [68]. These studies suggest a role of AhR-induced Th17 in promoting allergic or inflammatory airway diseases, but there are interesting differences between human and mouse T cells. These differences suggest that the response to AhR activation may vary according to cell type, maturation, and differentiation process.

3. Conclusion

We reviewed studies on the relationship between AhR function and airway inflammation, as it is important in the initial phase of asthma/COPD. In addition to studying the toxicological effects, we wish to promote studies focused on the immune regulation of endogenous AhR pathways. Moreover, it seems increasingly apparent that AhR acts by competing with other nuclear receptors in a complex manner. Further investigation may yield a novel treatment strategy for AhR-associated lung diseases.

Abbreviations

PCBs:	Polychlorinated biphenyls
B[a]P:	Benzopyrene
COPD:	Chronic obstructive pulmonary disease
TGF-α:	Transforming growth factor alpha
AhR:	Arylhydrocarbon receptor
ARNT:	AhR nuclear translocator
DRE:	Dioxin response element
CYP1A1:	Cytochrome P450 1A1
TCDD:	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
ROS:	Reactive oxygen species
TNF-α:	Tumor necrosis factor alpha
MMP:	Matrix metalloproteases
COX-2:	Cyclooxygenase-2
PGE_2:	Prostaglandin E_2
PGD_2:	Prostaglandin D_2
MUC5AC:	Oligomeric mucus/gel forming
BAL:	Bronchoalveolar lavage
PPARs:	Peroxisome proliferator-activated receptors
PPRE:	PPARs response element
IgE:	Immunoglobulin E
G-CSF:	Granulocyte colony-stimulating factor
FGF:	Fibroblast growth factor
EGF:	Epidermal growth factor
PDGF:	Platelet-derived growth factor.

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Review Article

Cholinergic Regulation of Airway Inflammation and Remodelling

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Acetylcholine is the predominant parasympathetic neurotransmitter in the airways that regulates bronchoconstriction and mucus secretion. Recent findings suggest that acetylcholine regulates additional functions in the airways, including inflammation and remodelling during inflammatory airway diseases. Moreover, it has become apparent that acetylcholine is synthesized by nonneuronal cells and tissues, including inflammatory cells and structural cells. In this paper, we will discuss the regulatory role of acetylcholine in inflammation and remodelling in which we will focus on the role of the airway smooth muscle cell as a target cell for acetylcholine that modulates inflammation and remodelling during respiratory diseases such as asthma and COPD.

1. Introduction

Acetylcholine is classically viewed as a neurotransmitter that regulates cognitive and behavioural functions in the brain, autonomous ganglionic transmission, and parasympathetic postganglionic transmission. In the respiratory tract, acetylcholine is the predominant parasympathetic neurotransmitter and its role in the regulation of bronchomotor tone and mucus secretion from airway submucosal glands is well established [1]. More recent findings suggest that acetylcholine regulates additional functions in the respiratory tract, including inflammation and remodelling during inflammatory lung diseases [2–4]. Moreover, it has become apparent that acetylcholine is synthesized by nonneuronal cells and tissues, particularly inflammatory cells and the airway epithelium [5–7]. These cells also express receptors for acetylcholine, including muscarinic receptors and nicotinic receptors that modulate inflammatory responses [2, 6]. Collectively, these findings have questioned the traditional view on the physiological and pathophysiological role of acetylcholine, which has opened up new possibilities for therapeutic targeting of the pulmonary cholinergic system. In this paper, we will discuss these recent findings in which we will focus on the role of the airway smooth muscle cell as a target for acetylcholine in inflammation and remodelling during respiratory diseases such as asthma and COPD.

2. The Origin of Acetylcholine

Acetylcholine is biosynthesized from choline and acetyl-CoA by choline acetyltransferase (ChAT) or carnitine acetyltransferase (CarAT) by several cell types in the respiratory tract [6]. Airway neurons and airway epithelial cells express ChAT and have been demonstrated by HPLC detection to release acetylcholine [5]. The release of acetylcholine from other nonneuronal tissues in the respiratory tract is suggested by the fact that also macrophages, mast cells, fibroblasts, smooth muscle cells, lymphocytes, and granulocytes express ChAT immunoreactivity [6]; however the release of acetylcholine from these cells and tissues has not yet been measured directly in the respiratory tract. Acetylcholine exerts its functions either via muscarinic receptors, a class of G-protein-coupled receptor subtypes, or via nicotinic receptors, a class of ligand-gated cation channels [8]. Most structural cells and inflammatory cells that are present in the respiratory system, including smooth muscle cells, fibroblasts, epithelial cells, mast cells, granulocytes, lymphocytes, and macrophages, express muscarinic and/or nicotinic receptors [2, 6]. For

a detailed overview of individual receptor subtypes and subunits expressed by these cells, we refer to a recent excellent overview by Wessler and Kirkpatrick [6]. The expression of muscarinic and nicotinic receptors, the expression of synthesizing enzymes such as ChAT, and the direct measurement by HPLC detection of acetylcholine release from nonneuronal tissues and cell cultures are solid evidence for the existence of a nonneuronal cholinergic system in addition to the more established neuronal cholinergic system in the airways.

The processing of acetylcholine by nonneuronal cells and tissues is not yet described in full although, for airway epithelial cells, secretory mechanisms have been described. Airway epithelial cells express the high affinity choline transporter (CHT1) that is involved in choline uptake as well as the organic cation transporter (OCT) subtypes 1 and 2, which play a dominant role in the release of acetylcholine by airway epithelial cells [9, 10]. Furthermore, the expression of the vesicular acetylcholine transporter (VAChT) by epithelial cells has been reported suggesting that storage of acetylcholine in vesicles and release via the fusion of these vesicles with the plasma membrane, as occuring in neurons, may represent an additional mechanism for acetylcholine release by nonneuronal cell types [9, 10].

The breakdown of acetylcholine into acetic acid and choline is catalysed by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), also known as pseudocholinesterase. The functional expression of AChE by airway epithelial cells is evidenced by observations that acetylcholine concentrations in cell supernatants of airway epithelial cell cultures were enhanced by the pharmacological inhibitor of AChE, neostigmine [5]. Collectively, the above-mentioned observations indicate that both neurons and nonneuronal cells and tissues in the respiratory system express and release acetylcholine. The functional role of nonneuronal acetylcholine on the airway smooth muscle includes bronchoconstriction [11, 12]. Additionally, acetylcholine may modulate airway hyperresponsiveness and remodelling, including the regulation of airway smooth muscle growth and the regulation of airway inflammation that promotes hyperresponsivness and remodelling. This role for acetylcholine will be discussed in the following sections.

3. The Muscarinic Receptor: Acetylcholine as a Proinflammatory and Remodelling Mediator

Muscarinic receptors are expressed by most structural cells in the airway wall, including the airway smooth muscle and by inflammatory cells that are involved in the pathogenesis of obstructive airway diseases [2]. Muscarinic receptors appear to play a proinflammatory role on these cells, suggesting that inhibition of muscarinic receptor function may have anti-inflammatory effects in these diseases. Increased expression of muscarinic M_1 and M_3 receptors on airway structural cells and sputum cells of COPD patients has been reported [13, 14]. Likewise, reduced expression of the autoinhibitory M_2 receptor on airway neurons in asthma has been reported [1]. Both effects could contribute to enhanced acetylcholine release and function in these diseases. The proinflammatory role of acetylcholine via muscarinic receptors is discussed below.

3.1. Direct Effects of Acetylcholine on Airway Smooth Muscle. The airway smooth muscle expresses muscarinic M_2 and M_3 receptors roughly in a 4 : 1 ratio [15]. The muscarinic M_3 receptor represents a primary target of acetylcholine in the airways, involved in the regulation of bronchoconstriction [15–18]. In addition, muscarinic receptors regulate proliferative and proinflammatory functions of the airway smooth muscle. It was observed that coadministration of muscarinic agonists with epidermal growth factor (EGF) in human airway smooth muscle cells induces a synergistic proliferative stimulus. This effect was associated with sustained activation of p70 S6 kinase [19, 20], an effect mediated by G_q-derived $G_{\beta\gamma}$ subunits that activate phosphatidylinositol-3-kinase (PI3K) in concert with the EGF receptor [19, 21]. In line with these findings, muscarinic receptor agonists induce an increase in proliferation of airway smooth muscle cells in combination with platelet-derived growth factor (PDGF) [22], which is mediated by G_q-protein-coupled muscarinic M_3 receptors and appears to involve a synergistic inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) [23]. GSK-3 is a multitasking enzyme that regulates multiple signalling proteins and transcription factors involved in contractile protein expression and cell proliferation of airway smooth muscle [23–26].

Muscarinic-receptor-induced airway remodelling could also involve mechanical regulation as airway smooth muscle constriction results in airway epithelial cell compression and subsequent activation of EGFR phosphorylation in the airway epithelium [27]. Indeed, a recent clinical trial demonstrates that repeated methacholine inhalations cause airway remodelling in the absence of inflammation, characterized by collagen deposition and increased TGF-β1 expression [28]. It is not yet clear whether such effects could also directly regulate remodelling of airway smooth muscle; however, mechanical strain of airway smooth muscle regulates cell proliferation and contractile protein expression [29–31], an effect enhanced in the presence of carbachol [32]. Clearly, this hypothesis needs to be followed up in future studies.

Muscarinic receptors on airway smooth muscle cells could also play a profound role in regulating the immunomodulatory function of airway smooth muscle [33, 34]. Cholinergic stimulation with the muscarinic receptor agonist carbachol augments inflammatory gene expression in bovine tracheal smooth muscle in combination with cyclic stretch, which induces a synergistic increase in the expression of IL-6, IL-8, cyclo-oxygenase (COX) 1 and 2, and urokinase-type plasminogen activator (PLAU) [35]. It was recently demonstrated that the activation of muscarinic receptors also interacts with several cytokines and growth factors that play an important role in the pathogenesis of asthma and COPD, in particular with TNF-α, PDGF-AB and cigarette smoke to enhance their inflammatory response in airway smooth muscle cells [36]. Thus, muscarinic M_3 receptor stimulation of airway smooth muscle with methacholine induces IL-6 and IL-8 production and augments the release

of these cytokines induced by cigarette smoke extract [36]. Our unpublished data show that this effect is dependent on downstream signalling to PKC, which activates the IκBα/NF-κB and MEK/ERK1/2 pathways [37]. This indicates that acetylcholine may also play an important role in the immunomodulatory processes driven by human airway smooth muscle.

The functional importance of these *in vitro* findings is illustrated by our *in vivo* studies that indicate a protective role for tiotropium bromide, a long-acting muscarinic antagonist, in the progression of airway smooth muscle remodelling. Thus, guinea pigs challenged with allergen for 12 consecutive weeks developed increased airway smooth muscle mass, increased contractile protein expression, and increased airway smooth muscle contractility, which were partially to fully prevented by treatment with tiotropium bromide [38]. In part, these inhibitory effects may have been due to the anti-inflammatory properties of tiotropium as airway eosinophilia was almost completely reduced by treatment with this compound [39]. Mucus gland remodelling and MUC5A/C hypersecretion were also prevented [39]. These results indicate that acetylcholine plays an essential role in remodelling of the airway smooth muscle (Figure 1). These effects may be direct, as suggested by the *in vitro* studies mentioned above, or indirect, as illustrated below.

3.2. Additional Effects of Acetylcholine on Airway Remodelling. Airway smooth muscle cells are embedded in the airway wall, and bidirectional communication between the muscle layer and the cell types and matrix protein structures that surround the muscle bundle is key to the development of abnormalities in airway smooth muscle phenotype and function in obstructive airways disease [40]. Fibroblasts are key effector cells in the production of extracellular matrix proteins that surround the airway smooth muscle bundle in the adventitia and submucosa of the airway wall [41]. Fibroblasts express functional muscarinic M_2 and M_3 receptors (predominantly M_2 receptors with relatively fewer M_3 receptors) [42]. *In vitro*, the muscarinic agonists carbachol and oxotremorine cause an increase in (^{3}H)-thymidine incorporation (as a measure of cell proliferation) in human lung fibroblast cell lines and primary fibroblasts. This effect is mediated by the M_2 receptor and regulated by the MEK/ERK1/2 pathway [42, 43]. Tiotropium, a long-acting muscarinic antagonist, concentration-dependently inhibited ACh-induced proliferation of primary human fibroblast isolated from biopsies of lung fibrosis patients and myofibroblasts derived from these cells [44]. Furthermore, it was found that muscarinic agonists stimulate the incorporation of ^{3}H-proline into cellular proteins (as a measure of collagen synthesis) in human lung fibroblast cell lines and primary fibroblasts [45]. Also, tiotropium bromide inhibits collagen expression in the lung and small airways in guinea pigs repeatedly exposed to LPS [46]. Collectively, these studies support a role for acetylcholine in regulating fibroblast cell responses associated with remodelling.

A proinflammatory role of acetylcholine in fibroblasts was recently questioned by a study showing that the release of chemotactic mediators was not induced in fibroblasts incubated with acetylcholine because of a relative lack of M_3 receptor expression in these cells [47]. On the other hand, primary lung fibroblast cultures from surgical specimens of COPD patients treated with acetylcholine showed enhanced IL-8 and matrix metalloproteinase-2 release. This effect was mediated by muscarinic M_3 receptors [14], and tiotropium has an attenuating effect on metalloproteinase-2 production from lung fibroblasts induced by inflammatory stimulation [48]. It is possible that the enhanced expression of muscarinic receptors by fibroblasts of COPD patients explains the discrepancy between these two studies as Profita et al. [14] showed that muscarinic M_1 and M_3 receptor as well as ChAT expressions were increased in fibroblasts from COPD patients. Although the quantification of muscarinic receptor expression using antibodies should be approached with care [49], functional differences between healthy controls and COPD patients were also observed. In this study, acetylcholine induced a significant increase in the activation of the ERK1/2 and NFκB pathways in fibroblasts of patients with COPD and promoted cell proliferation to a greater extent than observed in fibroblasts of healthy controls [14]. These findings clearly indicate the function of fibroblasts in remodelling processes that occur in chronic inflammatory airway diseases but the proinflammatory role of lung fibroblasts in response to acetylcholine remains to be studied in further detail.

The airway epithelium is key to the development of airway inflammation and remodelling as it presents the first barrier to inhaled particles and allergens and regulates the secretion of proinflammatory cytokines. Epithelial damage during allergic airway inflammation plays a key role in asthma and exposes sensory nerve endings in the submucosa to the airway lumen, which promotes reflex mechanisms leading to enhanced vagal release of acetylcholine [40]. Moreover, the airway epithelium is predominant in its expression of ChAT and may present a direct source of nonneuronal acetylcholine [5]. Acetylcholine is a proliferative stimulus for human bronchial epithelial cells in culture in part by activation of muscarinic M_1 receptors [50, 51]. Acetylcholine also increased eosinophil, monocyte, and neutrophil chemotactic activity by bronchial epithelial cells [52, 53]. This effect probably involves muscarinic M_1 receptors that induce leukotriene B_4 release from epithelial cells, which in turn stimulates eosinophil, neutrophil, and monocyte chemotactic activities [52, 53]. Muscarinic receptor agonists also induced the release of prostanoids from airway epithelial cells. Thus, muscarinic M_3 receptors promote the activation of phospholipase A2, which stimulates the release of PGE_2 from isolated tracheae, but only in preparations with an intact epithelial layer [54]. In addition, a recent investigation in human bronchial epithelial cells showed that acetylcholine induces the production of IL-8, involving PKC, ERK1/2, and NFκB pathway activation via muscarinic receptors [55].

Collectively, these findings indicate that acetylcholine, derived from the vagal nerve and from nonneuronal origins such as the airway epithelium, may induce cell responses associated with airway wall remodelling and trigger proinflammatory cytokine release by structural cells of the airway wall, including airway epithelial cells, airway fibroblasts, and

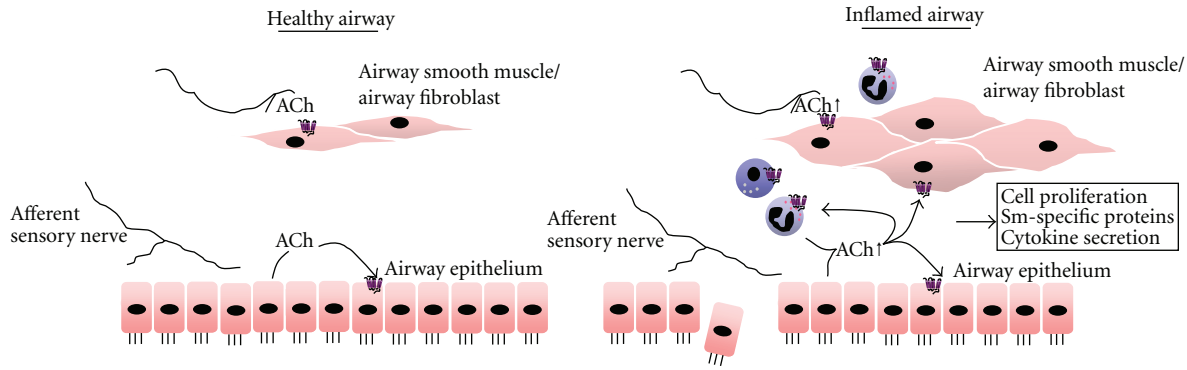

Figure 1: Muscarinic receptor regulation of airway inflammation and remodelling. In healthy airways, acetylcholine release from neuronal and nonneuronal origins release are limited. However, in response to environmental factors such as allergen or smoke, acetylcholine release is enhanced, which cooperates with proinflammatory cytokines and growth factors to induce airway smooth muscle and fibroblast cell responses including cell proliferation, smooth-muscle-specific protein expression, and the synthesis of chemokines and cytokines. As such, acetylcholine by acting on muscarinic receptors may contribute to both acute and chronic aspects of obstructive airways disease. Nicotinic receptors are expressed by airway structural cells and inhibit inflammatory cell activation; however, their role in regulating airway remodelling is largely unknown.

the airway smooth muscle itself. These mechanisms may promote airway inflammation and remodelling, including airway smooth muscle thickening.

3.3. Indirect Effects: Acetylcholine as a Proinflammatory Mediator. Airway inflammation in asthma and COPD likely plays an important role in the development of airway hyperresponsiveness and in the development of structural changes in the airway wall including increased airway smooth muscle mass. Inflammatory cells secrete cytokines and growth factors that induce a proliferative stimulus in airway smooth muscle cells (e.g., EGF, PDGF, and TGF-β) that may be amplified by the actions of acetylcholine as outlined above [56]. Moreover, acetylcholine, either from neuronal or nonneuronal origin, may regulate inflammatory cell responses in these diseases that explain the beneficial effects of anticholinergics on airway smooth muscle thickening [2].

The anticholinergic agent tiotropium bromide prevented allergen-induced airway eosinophilia in guinea pigs, indicating that muscarinic receptor signalling supports airway eosinophilia [39]. It has been demonstrated that muscarinic M_3 and M_4 receptors are expressed in human and guinea pig eosinophils; human eosinophils also appear to express the muscarinic M_5 receptor subtype [57]. However, Verbout et al. found an inhibitory effect of these muscarinic receptors on eosinophil activation [58]. Atropine, a nonselective muscarinic receptor antagonist, significantly potentiated antigen-induced eosinophil activation and airway hyperreactivity by increasing major basic protein deposition in the airways [58]. The inhibitory effect of muscarinic recepors on eosinophil activation in antigen-challenged animals is mediated by their suppressive effect on excitatory nerve growth factor (NGF) pathway [59]. The effect of muscarinic receptors on airway structural cells (epithelial cells, fibroblasts, airway smooth muscle cells) as outlined above may account for this discrepancy as proinflammatory cytokine production by these cells, including the release of eosinophil chemotactic activity, is enhanced by muscarinic receptor stimulation.

Muscarinic receptors are also expressed by macrophages and neutrophils and appear to play an important proinflammatory role in these cells. Muscarinic M_3 and M_5 receptors are expressed by macrophages, and muscarinic receptor agonists, such as carbachol, induce an increase in intracellular calcium and promote chemotaxis of these cells [60]. Alveolar macrophages also appear to express muscarinic M_1, M_2, and M_3 receptor subtypes [13]. Stimulation by acetylcholine of these cells induces the release of leukotriene B_4, which promotes neutrophil chemotaxis. This contention is in agreement with a study showing that, in bovine alveolar macrophages, muscarinic M_3 receptors induce the release of leukotriene B_4 [61]. Furthermore, it was recently shown that human alveolar macrophages respond to acetylcholine with the release of chemotactic activity for granulocytes, an effect likely involving leukotriene B_4 release [47]. The anticholinergic agent tiotropium suppressed the secretion of leukotriene B_4 by more than 70% after acetylcholine stimulation [47].

Treatment with tiotropium bromide significantly reduced airway inflammation and the Th2 cytokine production in bronchoalveolar lavage fluid (BALF) in both acute and chronic models of asthma. The levels of TGF-ß1 in BALF, the goblet cell metaplasia, thickness of airway smooth muscle, and airway fibrosis were all significantly decreased in tiotropium bromide-treated mice as well [62]. Tiotropium also concentration-dependently inhibited neutrophilic inflammation in response to cigarette smoke. Furthermore, the cigarette-smoke-induced pulmonary release of leukotriene B(4), interleukin-6, keratinocyte-derived chemokine, monocyte chemotactic protein-1, macrophage inflammatory protein-1 alpha and -2, and tumour necrosis factor alpha was dose-dependently reduced in murine model of COPD [63]. Neutrophil-elastase-induced goblet cell hyperplasia and gastrointestinal reflux-induced pulmonary inflammation can also be prevented by tiotropium treatment [64, 65]. These findings collectively indicate that acetylcholine, for example, nonneuronal acetylcholine derived

from the inflammatory cells themselves, promotes inflammatory responses in the airways via muscarinic receptors.

4. The Nicotinic Receptor: Acetylcholine as an Anti-Inflammatory Mediator

The airway smooth muscle also expresses nicotinic receptors including the $\alpha 3$ and $\alpha 7$ nicotinic receptor subtypes [6]. The role of the nicotinic receptor in airway smooth muscle is currently largely unknown. However, in sharp contrast to the proinflammatory role of muscarinic receptor stimulation, nicotinic receptors appear to play an important anti-inflammatory role in many cell types and organs. Nicotinic receptors are found in the airways on parasympathetic nerves, macrophages, eosinophils, neutrophils, mast cells [66–70], lymphocytes [71–73], airway smooth muscle cells [74], epithelial cells [75], and fibroblasts [76].

Acetylcholine from neuronal or nonneuronal origin can induce an anti-inflammatory effect via $\alpha 7$ nicotinic receptors in various models of acute inflammation [77, 78]. This is also established in models of pulmonary inflammation including a mouse model of hypersensitivity pneumonitis [79], asthma [80, 81], and inflammation following influenza infection [82, 83]. These *in vivo* findings are supported by *in vitro* findings showing that stimulation of the $\alpha 7$ nicotinic receptor in murine macrophage cell lines results in inhibition of LPS-induced TNF and HMGB1 release [84–86]. Moreover, acetylcholine and nicotine receptor agonists exert a strong inhibitory effect on the release of TNF-α and other cytokines such as IL-6, IL-1β, IL-12, IL-18, and IFN-γ without affecting the production of anti-inflammatory cytokines although in some cases upregulation of IL-10 production is observed [79, 87–89]. Acetylcholine anti-inflammatory properties are regulated by $\alpha 7$ nicotinic receptor on macrophages, because macrophages from $\alpha 7$-subunit-nicotinic-receptor-deficient mice failed to show inhibition of TNF-α release [66]. Local administration of GTS-21 (a selective $\alpha 7$ cholinergic receptor agonist) also inhibits TNF-α release in the mouse lung during LPS-induced inflammation [90].

In addition to exerting anti-inflammatory effects on macrophages, activation of $\alpha 7$ nicotinic receptors on endothelial cells inhibits TNF-α-induced expression of intercellular adhesion molecule-1 and chemokines IL-8, RANTES (regulated on activation, normal T cell expressed and secreted), and monocyte chemoattractant protein-1 [91], thereby preventing migration of inflammatory cells from the blood to the tissues. Systemic administration of nicotine or a selective $\alpha 7$ agonist also attenuates acid-induced lung injury by reducing TNF-α and MIP-2 concentrations and by reducing neutrophil accumulation in the airspaces of the lung in rats, resulting in decreased pulmonary oedema and pulmonary inflammation [67]. Paradoxically, profibrotic, and proinflammatory effects of nicotine have also been reported, as nicotine appears to promote fibronectin deposition by fibroblasts [92]. Nonetheless, most reports point to an anti-inflammatory and antiremodelling role for the $\alpha 7$ nicotinic receptor. Thus, nicotinic agonists, including acetylcholine, can limit cytokine release and tissue inflammation. It was recently shown that $\alpha 7$ nicotinic receptors stimulation of alveolar macrophages and neutrophils also reduced chemokine production including MIP-2, transalveolar neutrophil migration, and LPS- and *E. coli*-induced acute lung injury in the airways of mice [67]. Collectively, these data indicate that acetylcholine may exert potent anti-inflammatory effects in the lungs, primarily via $\alpha 7$ nicotinic receptors. Although expressed by airway smooth muscle, the role of the $\alpha 7$ nicotinic receptor is currently unknown. Clearly, experiments to identify the role of the $\alpha 7$ nicotinic receptor in airway smooth muscle, including its role in remodelling and in the immunomodulatory function of airway smooth muscle, are warranted.

5. Conclusion

Acetylcholine is the predominant parasympathetic neurotransmitter in the airways and an autocrine or paracrine hormone. Many structural and inflammatory cells, notably the airway epithelium, express and secrete acetylcholine and respond to acetylcholine (either neuronal or nonneuronal) via muscarinic and nicotinic receptors. The airway smooth muscle is of major importance to the physiological and pathophysiological actions of acetylcholine, which induces bronchoconstriction, airway smooth muscle thickening, and the modulation of cytokine and chemokine production by these cells (Figure 1). Additionally, muscarinic receptors regulate proinflammatory and remodelling responses of fibroblasts and airway epithelial cells and promote the release of leukotriene B_4 and other chemotactic mediators from macrophages and epithelial cells, resulting in eosinophil and neutrophil chemotactic activity.

In contrast to this proinflammatory role, nicotinic receptors expressed by inflammatory cells and structural cells exert potent anti-inflammatory effects, in which the $\alpha 7$ nicotinic receptor appears to play a central role. This receptor subtype can be targeted both by neuronal and nonneuronal acetylcholine and may present a useful therapeutic target for treatment. The role of the $\alpha 7$ nicotinic receptor in airway smooth muscle, and in airway remodelling in asthma and COPD, is currently largely unknown but clearly warrants future investigation. In addition, it is essential to design future studies to identify the (patho)physiological basis for the clear discrepancy between nicotinic and muscarinic receptor subtypes in the regulation of inflammation and remodelling.

Clearly, the airway cholinergic system holds excellent therapeutic potential. Muscarinic receptor antagonists, currently widely used as bronchodilators for the treatment of COPD, may have beneficial anti-inflammatory and antiremodelling effects. Although direct evidence for this assumption is lacking in asthma and COPD patients, treatment with the anticholinergic agent tiotropium reduces exacerbation frequency in COPD patients and reduces lung function decline in GOLD stage II COPD patients [93, 94]. These clinical findings are consistent with anti-inflammatory and remodelling effects of tiotropium, but proof for this hypothesis still needs to be obtained. In addition, the anti-inflammatory effects of the $\alpha 7$ nicotinic receptor suggest

that agonists for this receptor subtype are a strategy worth pursuing for the treatment of asthma and COPD.

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Review Article

In Vivo Computed Tomography as a Research Tool to Investigate Asthma and COPD: Where Do We Stand?

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Computed tomography (CT) is a clinical tool widely used to assess and followup asthma and chonic obstructive pulmonary disease (COPD) in humans. Strong efforts have been made the last decade to improve this technique as a quantitative research tool. Using semiautomatic softwares, quantification of airway wall thickness, lumen area, and bronchial wall density are available from large to intermediate conductive airways. Skeletonization of the bronchial tree can be built to assess its three-dimensional geometry. Lung parenchyma density can be analysed as a surrogate of small airway disease and emphysema. Since resident cells involve airway wall and lung parenchyma abnormalities, CT provides an accurate and reliable research tool to assess their role in vivo. This litterature review highlights the most recent advances made to assess asthma and COPD with CT, and also their drawbacks and the place of CT in clarifying the complex physiopathology of both diseases.

1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are the most common airway diseases worldwide and affect millions of people, with an increasing incidence. Asthma is characterized by a reversible airway obstruction and a bronchial hyperreactivity in response to a stimulus. Conversely COPD is defined as a chronic airway obstruction which is progressive and poorly reversible [1, 2]. Both diseases are associated with environmental factors such as allergens, viruses, bacteria, or toxics leading to an inflammatory response in patients genetically susceptible. Airway inflammation triggers oedema and bronchial wall infiltration by resident cells [3] (neutrophils, macrophages, mast cells [4], and eosinophils in asthma). Chronic inflammation leads to airway remodelling, and despite similarities, many clinical and pathological features show that the two diseases are distinct [5, 6]. The epithelium appears to be more fragile in asthma, and the epithelial membrane thickness and the bronchial smooth muscle are thicker than in COPD. Emphysema does not occur in asthmatic nonsmoker. In COPD, the epithelium displays mucous metaplasia, and inflammation is associated with loss of alveolar attachments, surrounded by peribronchial fibrosis [7]. Destruction of alveolar wall leads to emphysema which is a structural alteration seen in severe COPD. Beyond the immune system, asthma and COPD involve airway and lung parenchyma morphological changes, and computed tomography (CT) appears to be a noninvasive tool to investigate them in vivo [8, 9]. Submillimetric acquisition can be obtained with an isotropic voxel over the whole lung volume, and fully automatic quantification measurements are achievable using commercially available softwares. It provides an accurate research tool, suitable to help understand the complex physiopathology underlying the two diseases, which is still not well known [10]. Clinical, functional, and histological correlations have been reported. This article is focused on the most recent developments made the last decade to improve this technique, their findings and also their limits, and their perspectives.

2. Quantitative Measurement of Airways and Lung Parenchyma Using CT

2.1. Quantification of Airway Wall. The rationale of airway wall quantification using CT is the presence of an increased

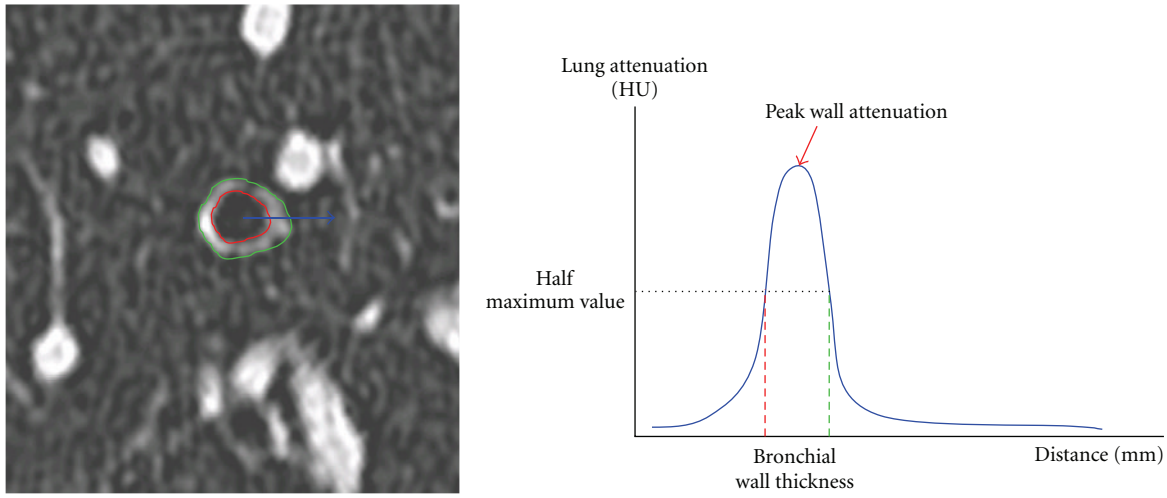


Figure 1: (a) Thin-section CT image perpendicular to the third generation of the right segmental apical bronchus, from a random patient. Red line indicates the external wall contour, and green line the internal layer. (b) Theoric single intensity curve (blue line) representing voxel attenuation variation along the blue arrow seen in right image. The bronchial wall thickness calculated with the FWHM principle is given by the difference between the two extreme values at which the mural portion attenuation is equal to half to its maximum (green and red dashed lines). According to Washko et al., the local Peak Wall Attenuation is given by the maximum attenuation value within the region of interest. Mean bronchial wall thickness and mean Peak Wall Attenuation shall be calculated using a circumferential integration of 128 one-dimensional rays, radiating outward the centroid of the bronchial lumen.

airway wall thickness in asthmatic and COPD patients compared with control subjects [11–15]. The airway intraluminal area (LA) and the total bronchial layer area (WT) are measured, in millimetres square. The wall area (WA) corresponds to the difference WA = WT − LA. WA% represents WA normalised on WT, that is, WA% = (WA/WT) × 100. WA and LA are not independent from body height, so they need to be normalised on body surface area (BSA), to reduce interindividual variation.

A manual method of segmentation has been described first [11]. It consists in tracing a manual region of interest around the internal and external bronchial wall, with a continuous extrapolation. This method is time consuming, exposed to intra- and interobserver variability and parallax error when the reconstructed plane is not strictly perpendicular to the bronchus main axis. Just a few numbers of bronchi divisions are reasonably available using this method, and the trunk of the right apical bronchus is the main target, owing to its geometry, nearly perpendicular to the axial plane.

Semiautomatic computational methods have been later developed to allow automated segmentation of the wall contours [12–14] and the bronchial tree [15, 16]. Briefly, active energy-driven contours are a region-based active contour model to extract the local image information. The full-width-at-half-maximum (FWHM) principle is given by the difference between the two extreme values at which the wall attenuation is equal to half to its maximum (Figure 1). The Laplacian-of-Gaussian algorithm is a function that combines a Laplace operator to detect edges as well as noise, and a convolution with a Gaussian kernel to smooth the image first. These algorithms have been used to segment airways wall contours, but none of them have demonstrated any superiority from each other. However, Brillet et al. have proven that they are not interchangeable in longitudinal studies [14].

Fetita et al. [15] and Montaudon et al. [16] have reported three-dimensional softwares to segment semiautomatically the bronchial tree (Figure 2). Perpendicular planes across the targeted bronchi can be acquired, and WA indices are automatically extracted. These softwares allow a fast and accurate postprocessing quantification, and this is relevant knowing the heterogeneity of alterations in asthma. However, the bronchial human tree displays a mean of 24 divisions including the trachea, and only 10 divisions are reasonably achievable using either manual or semiautomatic methods. Small conductive airways less than 1-2 mm diameter are not clearly visible on CT scans.

Another quantitative parameter has recently been assessed in both asthma and COPD: the bronchial wall attenuation [17–19]. Lederlin et al. [17], in a murine model of asthma, measured the peribronchial attenuation (PBA) using micro-CT with a spatial resolution of 46 microns. The manual method described in their study consisted in a manual segmentation of the peribronchial area, arbitrarily equal to the radius of the target bronchi lumen. In COPD, Washko et al. [18] and Yamashiro et al. [19] studied the peak wall attenuation (PWA) value, extracted from bronchial wall single-intensity curves based on FWHM principle (Figure 1). The mean PWA was calculated by taking the mean peak attenuation along 128 one-dimensional mural rays, radiating outward from the centroid of the airway lumen, using a circumferential measure.

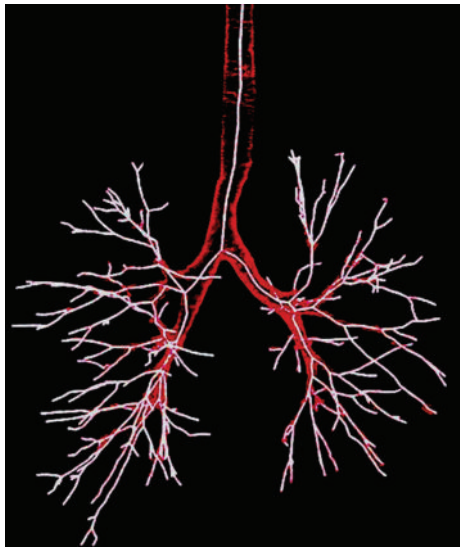


Figure 2: Bronchial tree volume automatically segmented using homemade dedicated software, extracted from a whole set of lung CT images. The skeleton of the bronchial tree is computed to obtain a simplified three-dimensional geometry of the bronchial tree.

2.2. Quantification of Lung Parenchyma. Small conductive and distal airways are beyond the spatial resolution of CT. Intralobular structures are not clearly visible, such as alveolar membranes, capillaries, or interstitial tissue. However, lung parenchyma density is a consequence of the X-ray attenuation by these lung structures, and any change in either of them may modify it. Therefore, lung attenuation provides an indirect tool to assess structural changes in distal airways, though it is nonspecific [20].

Lung alterations can be seen on CT images such as centrilobular micronodules, ground-glass opacities, mosaic pattern, air trapping, and emphysema and have been described in both pathologies [21, 22]. Quantification of these abnormalities has been studied through visual grading, but this method is potentially exposed to variability [23].

In asthma, Mikos et al. [24] measured air trapping on CT scans thanks to a manual method, at a window level of −600 HU and a window width of 1600 HU. Focal air trapping was assessed on end-expiratory scans, superimposing a 10 × 10 mm grid. The number of squares containing low lung attenuation was counted manually in every lung section. Diffuse air trapping was assessed as the ratio between mean lung density in expiration and inspiration (E/I ratio). Landmarks to match inspiratory and expiratory scans were placed at five levels, on superior margin of the aortic arch, tracheal carina, 1 cm below the carina, inferior pulmonary veins, and 2 cm above the diaphragm.

Two semiautomatic methods have been further developed [25, 26]. The rationale is the lower lung attenuation measured in emphysema and air trapping areas compared with normal areas. (a) The density mask technique [25] is based on a predefined voxel as a threshold to differentiate between areas of normal attenuation values, and areas of low attenuation (LAA). The density mask technique is defined as the percentage LAA% of total lung volume that contains voxels of lower attenuation values, usually lower than −960 UH in COPD to assess emphysema (Figure 3). (b) The percentile method [26] is based on predefined percentages (1%, 5%, 10%, and 15%) at which voxels have lower attenuation values (Figure 4).

Some drawbacks of these methods have been reported. Since lung attenuation values are not the same between different levels of radiation doses, CT manufacturers [27], or postprocessing softwares [28], Bakker et al. suggested that a calibration of air and blood should be performed before multicenter or longitudinal clinical trials and showed that normalisation of CT quantitative measurements by mean air attenuation value can reduce the variability.

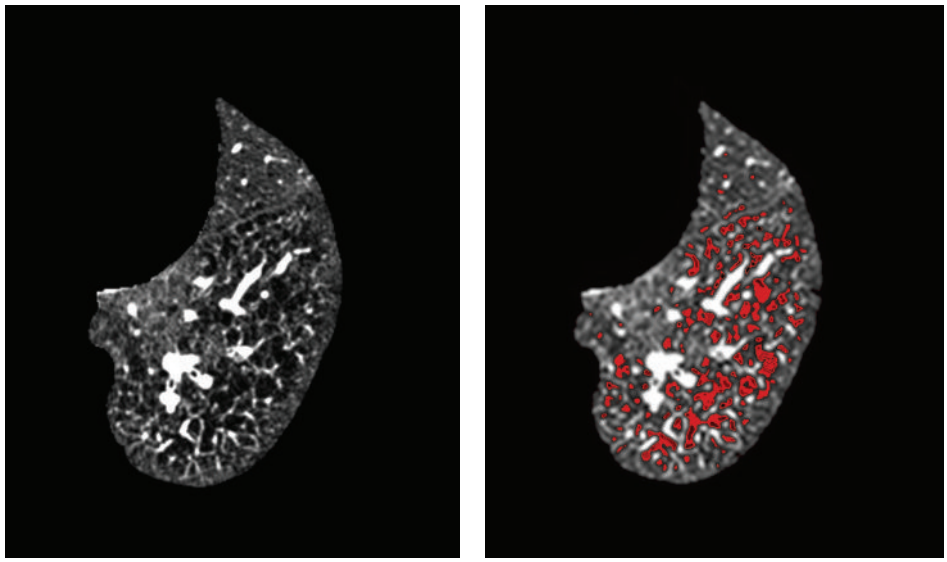
Age and lung volume involve variation of the voxel attenuation values, but not sex gender [29]. Densities are not the same on inspiration or expiration CT scans [30, 31]. The 15th percentile method has been reported to be more independent from lung volume changes than the density mask. Stoel et al. recommend adjusting the 15th percentile to the lung volume to reduce variability in followup studies [32].

Attenuation values are modified when CT is performed with or without contrast injection. Heussel et al. showed higher density in the lung parenchyma after contrast application. Therefore, the amount of emphysema may be underestimated, and they concluded that nonenhanced CT scans should be the reference [33].

3. Quantitative CT in Asthma

3.1. Large and Intermediate Airway Assessment in Asthma. Asthma involves both proximal and distal airways [1, 2]. Several studies have shown that airway wall thickness (WA) indices are increased in asthmatic patients compared with healthy volunteers [34–37]. According to histological data coming from autopsy studies of fatal cases, this may result from inflammatory changes such as oedema and infiltration of inflammatory cells, and structural changes such as an increased basal membranous thickness, smooth muscle cell layer and peribronchial fibrosis. From bronchial biopsies, Aysola et al. found that WA/LA ratio reflect increase in epithelial, and lamina reticularis thickness [38]. Montaudon et al. found that the slope and the maximal local slope of the WA/LA ratio both correlated with the subepithelial membrane thickness [39]. They showed that the bronchial geometric parameters correlated with smooth muscle area and with infiltration of the smooth muscle by mast cells.

The link between airway thickness measured on CT scans and bronchial reactivity (AHR) is controversial. The most common accepted theory is that part of the airway wall thickness is due to an increased smooth muscle cell layer, which is leading to AHR. Boulet et al. found a positive correlation between airway wall thickness and bronchial hyperreactivity, measured as a fall of 20% of forced expiratory volume in one second (FEV1) after a provocative concentration of metacholine [34]. However, Niimi et al. found that airway sensitivity was related to sputum eosinophil count but not to airway thickness. They also showed a negative correlation between airway thickness and bronchial reactivity, unrelated



(a) (b)

Figure 3: (a) Segmented thin-section CT image of basal left lung area in which lung contours and mediastinum were removed. (b) Same image using the density mask technique.

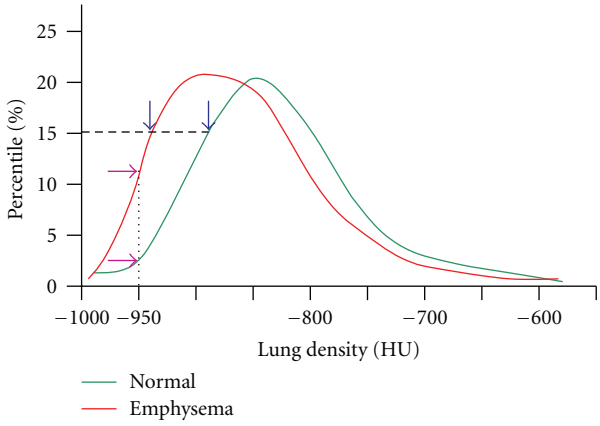


Figure 4: Theoric models of voxel attenuation frequencies in a normal subject (green curve) and emphysematous patient (red curve). The density mask technique is defined as the percentage of total lung volume that contains voxels lower than a predefined voxel index, usually −950 HU to assess emphysema (purple arrows). The percentile method is based on predefined percentages at which voxels have lower attenuation values. Blue arrows indicate the crossing points of green and red curves with the 15th percentile.

to eosinophil count. They concluded that airway walls are stiff when thickened, indicating that remodelled asthmatic airways are less distensible and may explain chronic airway obstruction [35]. In other studies, the same authors showed that WA indices are increased in severe as in mild-to-moderate patients with asthma compared with control subjects. In addition, they showed that WA indices correlate with the duration of disease, the severity, and the degree of airflow obstruction [36].

Data around intraluminal area (LA) are controversial too. Niimi et al. [36] and Aysola et al. [38] did not find any significant difference between asthmatic patients and controls. Lynch et al. reported that 77% of asthmatic patients had an internal bronchial diameter to pulmonary artery ratio >1.0, indicating bronchial dilatation [37]. Conversely, Montaudon et al. [39] and Beigelman-Aubry et al. [40] reported a bronchial cross-sectional area significantly smaller in asthmatic than in healthy volunteers. These different features may be explained by heterogeneity of bronchial diameters in asthma. For instance, Niimi et al. measured the right apical segmental bronchus, whereas Aysola et al. quantified the first to the third generation, and Montaudon et al. from the fourth to the tenth.

Peribronchial density has been recently assessed by Lederlin et al. in a murine model of asthma [17]. They did not quantified WA or LA, but micro-CT peribronchial density (PBA), and showed that the attenuation around the bronchial tree in asthmatic mice was increased compared with controls. This increase correlated with both inflammation and remodelling features.

CT bronchial dimensions have been studied to assess medication effects. Kurashima et al. used CT to evaluate the efficacy of inhaled corticosteroid and found a decrease in airway wall thickness among asthmatic patients with duration of symptoms less than 3 years, a minor response among 3 to 5 years and no change in wall thickness in patients with more than 5 years duration of disease [41]. However, Brillet et al. did not found any change of both WA and LA after a combination of salmeterol/flucitasone daily for 12 weeks, though patients displayed clinical and functional improvement, assessed by a decrease in FEV1 and expiratory reserve volume (ERV) [42].

3.2. Lung Parenchyma Assessment in Asthma. Asthma is a predominant airway diseases and does not involve lung

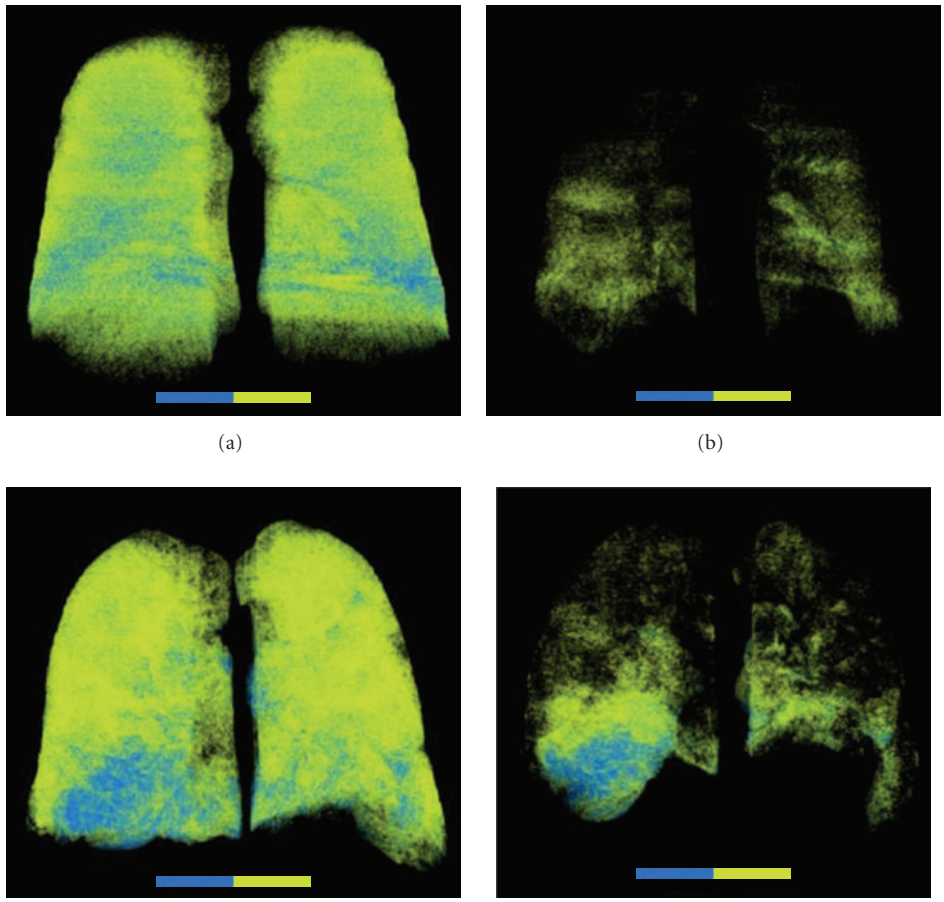


Figure 5: Ventral views of lung attenuation volume samples, computed using the density mask technique to extract voxels below −850 HU. Green areas are representative of voxels between −850 and −900 HU, and blue areas between −900 and −950 HU. Images (a) and (b) were acquired with spirometrically gated CT scans in a non-severe asthmatic subject, in inspiration (a) and in expiration (b). Same images were acquired at same levels of inspiration (c) and expiration (d) from a severe asthmatic subject.

parenchyma destruction during stable stages [1, 2]. However, CT lung parenchyma changes have been reported.

Using a visual grading, Laurent et al. observed that the mosaic perfusion pattern was significantly increased at full inspiration in 22 patients with stable moderate asthma (23%), compared with 12 healthy nonsmoker [21]. This result was addressed to either hypoxic vasoconstriction or small airway obstruction. They also found that air trapping was increased in asthmatic and healthy smokers, but not in controls. In asthmatic patients, air trapping scores correlated with FEV1 and FEF25–75%, and this was ascribed to small airway obstruction.

Mikos et al. using a manual method, showed that focal and diffuse air trapping (E/I ratio) correlated with airway wall thickness (WA%) [24]. Focal air trapping was significantly increased in a subgroup of 10 asthmatics patients with normal FEV1% predicted and FEV1/FVC%.

In a multivariate analysis of risk factors, Busacker et al. studied 60 patients with severe asthma, 34 nonsevere asthma and 26 controls. Using a semiautomatic method of CT quantification based on the density mask technique, he defined air trapping as areas of attenuation lower than −850 HU on CT scans acquired in expiration. Air trapping was considered significant whether more than 9.66% of the whole lung volume was involved (Figure 5). They analysed that patients with the air trapping phenotype are more likely to have a history of asthma-related hospitalizations and mechanical ventilation. Several risk factors of this phenotype where noted such as a history of pneumonia, neutrophilic inflammation, and atopy [43].

Mitsunobu et al. evaluated the heterogeneity of asthma disease using LAA% and a fractal analysis, to extract a D coefficient as a surrogate of small airway geometry complexity [44]. They found that LAA% and D correlated in a subset of asthmatic smokers, but not in nonsmokers. LAA% was different in mild and moderate asthma, but D was not. They concluded that D was a biomarker of emphysematous changes, which can help to characterize areas of low attenuation.

Lung density has been used to evaluate CT changes after therapy. Mitsunobu et al. demonstrated that mean lung density (MLD) and relative lung areas of attenuation under

−950 HU were improved after systemic glucocorticoid therapy [45]. MLD and LAA% under −950 HU both correlated with FEV1% improvement after therapy.

4. Quantitative CT in COPD

4.1. Large and Intermediate Airway Assessment in COPD. Small airways are the main site of obstruction in COPD [1, 2]. However, large airways are not free of abnormalities. Lee et al. defined a tracheal index (TI) as the ratio between the tracheal diameter measured on the coronal plane, and the sagittal diameter. They showed significant correlation between TI and severity of emphysema [46]. Sverzelatti et al. studied the prevalence of bronchial diverticula in smokers [47]. Grade 2 was defined as the presence of more than three diverticulas in large airways. This feature correlated with a more frequent history of cough, a greater extent of emphysema, a more severe bronchial wall thickening, and a heavier level of smoking.

Nakano et al. demonstrated that the mean dimensions of large and intermediate airways with an internal perimeter greater than 0.75 cm predicted the mean dimensions of small airways with an internal diameter of 1.25 mm [48].

Several studies have shown that airway wall thickness correlates with pulmonary function tests (PFTs) [49–53]. In a study conducted in 114 smokers, Nakano et al. showed that WA% measured on the trunk of the right apical bronchus correlated with FEV1 predicted, forced vital capacity (FRC), and residual volume/total lung capacity (RV/TLC) [49]. Grydeland et al. demonstrated that DLCO correlates with both emphysema and airway wall thickness [50], though Nakano et al. did not find significant correlation. Berger et al. measured airway dimensions with spirometrically gated CT. They showed that normalized WA and LA correlated with FEV1 and FEF25%–75% in smokers with and without COPD. Moreover, these dimensions were significantly larger in smokers with COPD than in smokers without COPD or non smokers [51].

Achenbach et al. wondered whether the strong correlations calculated between small airways dimensions and PFT is overestimated by the point spread function (PSF) artefact, or not. PSF involves blurring of the small airways contours and can lead to overestimate them using the FWHM algorithm. Using another three-dimensional approach taking into account the PSF, they assessed a median of 619 orthogonal airway locations per patient. They observed a significant correlation between airway dimensions and FEV1 in COPD patients. This correlation was higher from large to small airways, which is in agreement with FWHM principle [54].

Shimizu et al. have compared airway dimensions in 28 male COPD versus 12 sex and age-matched asthmatic and 13 age-matched healthy smokers. WA% and LA were measured from the 3rd to the 6th generation. At any generation, WA% was smaller and LA larger in COPD than in asthma, followed by controls. FEV1 predicted and FEV1/FVC was similar between asthma and COPD. They concluded that remodelling is more prominent in asthma than in COPD under stable clinical conditions [55].

Bronchial wall attenuation has been recently assessed in COPD. Washko et al. and Yamashiro et al. used the FWHM algorithm to extract the Peak Wall Attenuation value from the bronchial wall, as a surrogate of its main density. They showed strong correlations between this new biomarker of airway wall structural changes and airway obstructions assessed by PFT. Correlations were stronger in small airways. However, PWA extracted with FWHM principle is not independent from airway wall dimension [18, 19].

4.2. Lung Parenchyma Assessment in COPD. Ex vivo studies in isolated lungs and in vivo invasive measurements of airway resistance revealed that distal airways are the main site of airflow obstruction in COPD [5, 6]. Pathological studies highlighted that the small conductive airways are infiltrated by phagocytes (macrophages and neutrophils), dendritic cells, and T and B lymphocytes. Structural changes include airway wall thickness and obstruction by muco-inflammatory exudates and emphysema. Lung density provides an indirect tool to assess them in vivo, though non specific.

Centrilobular nodules and branching lines are areas of high attenuation and reflect pathological changes in small conductive airways, either inflammation or fibrosis. Destruction of alveolar walls is the hallmark of emphysema, and this pathological feature induces decreased areas of lung attenuation. Using the density mask and the percentile methods, Madani et al. showed that the −960 HU voxel index and the 1st percentile correlate with emphysema extent on pathological examinations [56]. Gevenois et al. showed that expiratory quantitative CT is not as accurate as inspiratory CT to measure lung emphysema [57].

However, areas of decreased attenuation can be visible on inspiratory images as a mosaic pattern, and air trapping on expiratory images. Both obstruction of the small conductive airways and loss of alveolar attachments are associated with destabilisation and premature airway closure during expiration. Therefore, differentiating emphysema from air trapping is not reliably achievable on CT images when assessed visually. Nevertheless, Matsuoka et al. have developed a quantitative method to evaluate it. They suggested that voxels <950 HU represent emphysema, and the relative volume changes, between inspiration and expiration, of voxels between −950 HU and −860 HU is thought to reflect air trapping [58–61].

Another paradoxical fall in lung density has been reported by Shaker et al. in COPD smokers. LAA% with a voxel index of −910 HU displayed a rapid fall in lung density after smoking cessation, mimicking rapid progression of emphysema. This was ascribed to an anti-inflammatory effect of smoking cessation and is not to be misinterpreted [62]. Persistent airway inflammation and emphysema progression have been showed in exsmokers after 4-year smoking cessation [63].

Correlations between the extent of emphysema and pulmonary function tests have been long reported [64–69]. COPD is characterized by expiratory airflow limitation that results in delayed emptying of the lung, poorly reversible. LAA% has been shown to correlate with FVC% predicted, FEV1% predicted, FEV1/FVC, RV/TLC, and DLCO/VA.

Gurney et al. studied emphysema distribution and showed that predominantly lower lobe zones of emphysema are more likely to correlate with obstructive dysfunction and DLCO [70].

CT quantification has been assessed as a predictor of lung function decline in smokers with normal PFTs. Tsushima et al. reported that abnormal CT findings were predictive of airflow limitation and development of emphysema in smokers with normal FEV1 [71]. Yuan et al. demonstrated that CT quantification of overinflation is predictive of FEV1 decline in smokers with normal lung function [72]. Using the 15th percentile, Hoesein et al. demonstrated that the extent of emphysema quantified by CT correlates with the lung function decline assessed by FEV1 at 3-year followup [73].

CT has been used to explore clinical outcomes associated with COPD. Mair et al. [74], and Ogawa et al. [75] reported a negative correlation between LAA% and body mass index (BMI), and emphysema dominant COPD phenotype showed stronger negative correlation with BMI than airway dominant. Ohara et al. reported that LAA% correlates with significant reduced bone density, as a biomarker of osteoporosis in COPD patients [76]. Independent associations with thoracic calcification have been reported by Dransfield et al., suggesting that LAA% can be used as a risk factor of cardiovascular disease in patients with and without COPD [77]. Emphysema is associated with an increased risk of lung cancer [78, 79]. Gullón et al. showed that presence of emphysema in patients with nonsmall cells lung cancer affect the survival rate and can be consider a prognostic factor [80]. Haruna et al. have followed up 251 COPD patients. Among them, 79 died, and 40 deaths were attributable to respiratory disease not involving lung cancer. A multivariate analysis comparing age, PFT, BMI, and emphysema assessed by CT revealed that LAA% had the strongest association with mortality [81].

Using CT quantification, Shaker et al. did not find significant correlation between CT emphysema quantification and emphysema progression after corticosteroid therapy [82]. Nevertheless, lung attenuation has been reported to be a predictor of outcome after lung volume reduction surgery in severe COPD. Wahsko et al. showed weak but statistically significant correlation between emphysema CT measures and 6-month postoperative outcomes assessed by FEV1 and maximal exercise changes [83]. Sciurba et al. demonstrated a poorer survival postoperative rate in patients with increased LAA%. Both studies did not show any significant correlation with airway wall thickness [84].

4.3. COPD Classification Using CT. According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guideline, COPD is a disease state characterized by airflow limitation that is not fully reversible. Irreversible airflow limitation is defined as FEV1/FVC < 70% after inhalation of β2-agonist. Chronic bronchitis and emphysema are not included in the GOLD definition, because they do not allow accurate discrimination based on clinical symptomatic features [85].

Nakano et al. suggested that CT is useful to discriminate between patients who have primarily parenchyma disease from those who have primarily airway pathology. Using WA% and LAA% as objective and quantitative surrogates of, respectively, airway and lung disease, they found that they could divide COPD patients into groups; airway remodelling-dominant group (high WA% and low LAA%), emphysema dominant group (low WA% and high LAA%), and a mixed group (high WA% and high LAA%). They did not study the clinical features associated with this classification based on morphological CT changes [86].

Fujimoto et al. classified COPD patients into three morphological groups and studied their clinical and functional signification. The A phenotype was defined as absence of emphysema with or without bronchial wall thickening, E phenotype as emphysema without wall thickening, and M phenotype as a combination of emphysema and bronchial wall thickening. They showed that the clinical features between these three phenotypes were different. The A phenotype showed a higher prevalence of nonsmoker COPD patients, higher BMI and DLCO, and milder hyperinflation compared with the E. The M phenotype showed a higher prevalence of severe COPD, assessed by sputum level, productive cough, wheezing, exacerbation, and hospitalizations. A and M phenotypes showed greater airflow reversibility of airflow limitation responsive to β2-agonist inhalation compared with E [87, 88].

Fujimoto et al. studied the efficacy of long-acting muscarinic antagonist (tiotropium) in COPD patients, classified in dominant emphysema or nondominant on their morphological CT scans. Tiotropium improved airflow limitation in all types, regardless to the emphysema dominance, and dynamic hyperinflation in the emphysema dominant phenotype [89].

5. Perspectives

Strong efforts have been made the last decade to assess CT as a research tool to understand the role of resident cells in asthma and COPD. This literature data screening show that clinical and animal study is now available in vivo thanks to MDCT technology. However, a cellular level of detection has not been reached yet. Further developments need to be performed to obtain cellular-specific quantifications.

Spatial resolution should be improved. Small conductive airways inferior to 1 mm are not clearly visible on CT images, and they still need to be addressed. An indirect CT parameter is provided by lung attenuation. Lung attenuation is though nonspecific and may be altered by any change in intralobular structures. Blurring effect may also modify CT wall thickness quantification of small to intermediate airways. A better delineation of these structures may allow a better understanding of their alterations in asthma and COPD.

Wall density is a new biomarker in asthma and COPD which need further development. Only nonenhanced wall attenuation has been reported. No data exists about CT-enhanced wall attenuation value changes. For instance, fibrosis is typically characterized in organs by a delayed enhancement on CT scans using an iodine contrast medium, and data around peribronchial fibrosis in COPD or chronic asthma

have not been reported. Specific contrast medium should be developed to enhance target cells, and density may be the one tool to quantify possible enhancement.

Postprocessing softwares are to be improved. Just a few numbers of bronchial generations are available for 3D segmentation. The gradient of density between bronchial and parenchyma air attenuation is not suitable for an accurate segmentation beyond a few number of bronchi generations.

MRI techniques using noble hyperpolarized gases have been reported, but they are cost effective and not suitable for a routine clinical or research practice widely used [90–92]. To our knowledge, no data exists about proton MRI of bronchi wall quantification. Enhanced MRI using gadolinium medium or others in bronchi wall is not documented. Due to its inocuity, MRI should provide cine images of bronchi diameter modifications under physiologic or pathologic conditions.

6. Conclusion

CT is an accurate tool to investigate in vivo asthma and COPD physiopathology. Quantification of airway wall and lung parenchyma has demonstrated strong correlations with clinical, functional, and pathological features, in humans or in animal models. However, spatial resolution and specific contrast medium need to be further developed to allow a cellular level of detection which has not been reached yet.

Abbreviations

COPD:	Chronic obstructive pulmonary disease
CT:	Computed tomography
LA:	Luminal area
WT:	Wall layer area
WA:	Wall area
BSA:	Body surface area
MLD:	Mean lung density
FWHM:	Full-width-at-half-maximum
PWA:	Peak wall attenuation
HU:	Hounsfield Unit
LAA:	Lung attenuation area
AHR:	Airway hyper reactivity
PFT:	Pulmonary function testing
FEV1:	Forced expiratory volume in 1 second
ERV:	Expiratory residual volume
FEF25%–75%:	Forced expiratory flow 25%–75%
FVC:	Forced vital capacity
FRC:	Forced residual capacity
RV:	Residual volume
TLC:	Total lung capacity
DLCO:	Diffusing capacity of the lung for carbon monoxide
TI:	Tracheal index
PSF:	Point spread function
BMI:	Body mass index
MDCT:	Multidetector computed tomography
MRI:	Magnetic resonance imaging.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Review Article

The Pivotal Role of Airway Smooth Muscle in Asthma Pathophysiology

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Asthma is characterized by the association of airway hyperresponsiveness (AHR), inflammation, and remodelling. The aim of the present article is to review the pivotal role of airway smooth muscle (ASM) in the pathophysiology of asthma. ASM is the main effector of AHR. The mechanisms of AHR in asthma may involve a larger release of contractile mediators and/or a lower release of relaxant mediators, an improved ASM cell excitation/contraction coupling, and/or an alteration in the contraction/load coupling. Beyond its contractile function, ASM is also involved in bronchial inflammation and remodelling. Whereas ASM is a target of the inflammatory process, it can also display proinflammatory and immunomodulatory functions, through its synthetic properties and the expression of a wide range of cell surface molecules. ASM remodelling represents a key feature of asthmatic bronchial remodelling. ASM also plays a role in promoting complementary airway structural alterations, in particular by its synthetic function.

1. Introduction

The pathophysiology of asthma is characterized by the association of airway hyperresponsiveness (AHR), inflammation, and remodelling [1–3]. AHR is defined by an increased airway narrowing to a wide range of stimuli and is responsible for recurrent episodes of wheezing and breathlessness. Airway smooth muscle (ASM) is considered as the main cell type involved in AHR [4, 5]. Bronchial inflammation in asthma involves the recruitment of various inflammatory cells including eosinophils, mast cells and T lymphocytes [1]. However, the microlocalization of these cell types is different within the asthmatic ASM layer [6], suggesting complex interactions between inflammatory cells and ASM cells. Bronchial remodelling is described as an increased thickening of the bronchial wall due to various structural alterations including epithelial changes [7], subepithelial membrane thickening, enhanced extracellular matrix (ECM) deposition [8], mucous gland and goblet cell hypertrophy and hyperplasia [9], neoangiogenesis [10], and increase in ASM mass [11, 12]. This latter appears to be a key feature of bronchial remodelling since increased ASM mass is associated with a decrease in lung function in asthma [13–15]. The aim of the present article is thus to review the pivotal role of ASM in the pathophysiology of asthma.

2. Role of ASM in Airway Hyperresponsiveness

AHR is usually described as either nonspecific or specific AHR (Table 1). Nonspecific AHR is a common feature of asthma, although it is also found in some patients suffering from chronic obstructive pulmonary disease or allergic rhinitis [16]. Stimuli inducing nonspecific AHR can be direct or indirect (Table 1). Direct mediators stimulate ASM cell membrane receptors. For instance, methacholine activates muscarinic M3 receptor and induces ASM contraction [17]. Conversely, indirect mediators first stimulate one or more intermediary cells, leading to the release of contractile agonists, which, in turns, induce ASM contraction [18]. All of these indirect challenges are associated with the release

Table 1: Stimuli used to assess airway hyperresponsiveness (AHR) *in vivo*.

AHR	Mechanisms	Types	Stimuli
Nonspecific	Direct	Pharmacological	Methacholine [22]
			Histamine [23]
Nonspecific	Indirect	Physical	Exercise [24]
			Cold air, dry air [25]
			Eucapnic hyperventilation [26]
			Hypo osmolar solution [27]
Nonspecific	Indirect	Chemical	Adenosine monophosphate [28]
			Mannitol [27]
Specific	Indirect	Allergen	Pollens, House dust mites [29]

Table 2: Contractile and relaxant mediators for ASM.

Types of mediators	Actions	Stimuli
Inflammatory	Contraction	Histamine [23]
		Tryptase [42]
		Prostanoids (PGF2α, PGD2, TXA2) [43]
		Cysteinyl leukotrienes (LTC4, LTD4, LTE4) [22]
		Endothelin [44]
Neurotransmitters	Contraction	Acetylcholine [45]
		Neurokinine A [46]
		Substance P [46]
		Calcitonin Gene Related Peptide [47]
Inflammatory	Relaxation	Prostanoids (PGE2, PGI2) [48]
Neurotransmitters	Relaxation	Adrenaline [49]
		Noradrenaline [50]
		Vasoactive Intestinal Peptide [46]

of mast cell mediators, such as prostaglandins, PGD2 and PGF2α, or histamine, which, respectively, activate ASM cell membrane receptors TP, FP, and H1 [19]. For instance, acute and intense exercise induces airway dehydration, increasing osmolarity of the airway surface liquid [20]. Cell volume shrinkage and restoration release both mast cell mediators and acetylcholine from nerves [20]. On the other hand, specific AHR is limited to allergic asthmatics. In such case, the stimulus is an allergen against which the patient is specifically sensitized. The mechanism of ASM contraction also involves the release of mast cell mediators. Specific AHR measurement is restricted to clinical trials or detection of occupational diseases. Indeed, allergen-specific stimulation can enhance nonspecific AHR [21] and induce a late phase response [19].

The mechanisms of AHR in asthma may involve (i) a larger release of contractile mediators, (ii) a lower release of relaxant mediators, (iii) an improved ASM cell excitation/contraction coupling, and/or (iv) an alteration in the contraction/load coupling.

2.1. Larger Release of Contractile Mediators. The contraction of ASM can be induced by a variety of extracellular messengers, which act through interaction with specific receptors at the site of the plasma membrane of ASM (Table 2). These contractile agonists, either inflammatory mediators or neurotransmitters, are released at high concentrations in asthmatic bronchi, especially regarding mast cell mediators following indirect challenges [19]. For instance, mast cells release tryptase, which increases ASM cells cytosolic calcium concentration [30], allowing them to contract after activation of the protease activated receptor (PAR) type 2, expressed on their membrane [31]. Mast cells, but also eosinophils, produce contractile prostaglandins such as PGF2α, PGD2, and thromboxane TXA2. The concentration of these prostaglandins, and also that of histamine, is increased in asthma [32]. Similarly, inflammatory cells also produce both cysteinyl leukotrienes (LTC4, LTD4, LTE4), which are also very potent direct contractile agonists of ASM [33], and LTB4, which acts indirectly through the release of TXA2 [34]. Such cysteinyl leukotrienes are also increased in asthma [35], as well as endothelin [36], another direct contractile agonist of ASM [37], produced by epithelial cells or endothelial cells [38]. The release of contractile neurotransmitters, acetylcholine or tachykinins, is also known to be higher in asthma [39, 40]. It is noteworthy that the loss of epithelial barrier integrity commonly associated with asthma may increase exposure of ASM to inhaled contractile agonists [41].

2.2. Lower Release of Relaxant Mediators. AHR may also be related to a lower release of relaxant agonists either neurotransmitters or inflammatory mediators (Table 2). Physiologically, the adrenergic innervation is sparse in the ASM [51]. Moreover, a decreased number of β2-adrenergic receptor sometimes associated with its desensitization has been suggested in asthma following chronic medication with short-acting β2-agonists [52]. A lower release of adrenaline has also been demonstrated during asthma attacks [53]. The second important relaxing neurotransmitter is the vasoactive intestinal peptide (VIP), which is colocalized with acetylcholine [54]. However, the expression of VIP in the asthmatic ASM is not decreased but surprisingly increased [55]. By contrast, the production of PGE2, which is known to relax ASM, is significantly lower in asthmatic than in nonasthmatic ASM cells [56]. Other cellular sources of PGE2 and other ASM relaxant, such as NO, may also be implicated.

2.3. Improved ASM Cell Excitation/Contraction Coupling. Cytokines, such as IL-5 or IL-13, that are both increased in asthma, do not contract ASM *per se*. In fact, IL-5 induces

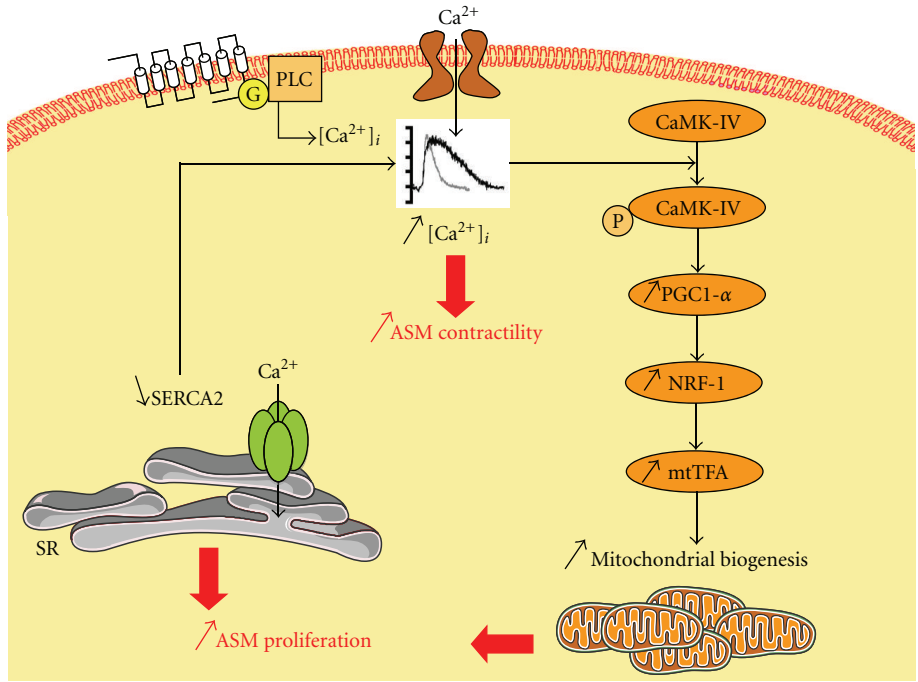


Figure 1: Impaired ASM cell calcium homeostasis leading to enhanced proliferation in asthma. In severe asthmatic ASM cells, an altered calcium homeostasis related to an increased influx leads to phosphorylation and activation of CaMK-IV, that, in turns, successively activates PGC-1α, NRF-1, and mtTFA. This transduction pathway results in an increase of mitochondrial biogenesis leading to enhanced ASM proliferation [61]. In nonsevere asthmatic ASM cells, an altered expression and function of SERCA2 may account for the altered calcium homeostasis, which leads to enhanced ASM proliferation [62]. Whatever the mechanism, such altered calcium homeostasis enhances cell contractility. ASM: airway smooth muscle; CaMK-IV: calcium/calmodulin-dependent protein kinase IV; G: G protein; mtTFA: mitochondrial transcription factor A; NRF: nuclear respiratory factor; PGC: peroxisome proliferator-activated receptor γ coactivator; PLC: phospholipase C; SERCA: sarcoendoplasmic calcium pomp; SR: sarcoplasmic reticulum.

in vitro AHR to acetylcholine in isolated rabbit ASM tissue and IL-13 reduces responsiveness to adrenaline in human ASM cells [57, 58]. Moreover, mast cell-derived tryptase induces AHR to histamine *in vitro* using human bronchi from actively sensitized [59] or nonsensitized patients [60] possibly *via* its enzymatic activity. However, such AHR occurs after an initial calcium response and, thus, in the absence of any spontaneous contraction. Indeed, AHR may also depend on excitation/contraction coupling, which associates two subsequent steps within the ASM cell, that is, (i) the calcium response induced by extracellular messengers and (ii) the calcium sensitivity of the contractile apparatus.

On the one hand, following extracellular stimulation, ASM cytosolic calcium homeostasis is dependant on various components. For instance, the spontaneous return to baseline of the cytosolic calcium concentration is dramatically delayed in asthmatic ASM cells [61]. Such a delay can be related with an abnormal calcium entry [61] and/or with a downregulated expression and function of type 2 of the sarcoendoplasmic calcium pump, that is, SERCA2 [62] (Figure 1). SERCA2 protein expression could be experimentally decreased by IL-13 or TNF-α (TNFSF2) [63]. Alternatively, the proinflammatory cytokine, IL-1β, can increase CD38 (ADP-ribosyl cyclase) expression. Then, CD38 increases cyclic ADP-ribose (cADPR) production, which, in turns, activates ryanodin receptor (RyR), leading to an increase in cytosolic calcium concentration [64–66]. In addition, calcium compartmentalization could be another determinant of airway responsiveness. In asthmatic ASM, there could be a greater proportion of intracellular calcium within the deep cytosolic space as compared to the superficial subplasmalemmal space [67], leading to AHR. However, such hypothesis needs to be confirmed.

On the other hand, the calcium sensitivity of the contractile apparatus can be increased, leading to a higher contractile response after a similar ASM intracellular calcium rise [68]. Two different mechanisms have been described according to the dependency or the independency from the myosin light chain kinase/myosin light chain phosphatase (MLCK/MLCP) ratio (Table 3). Regarding MLCK/MLCP-dependent AHR, the level of MLCK is increased in asthmatic ASM [69, 70]. The transcription factor CCAAT/enhancer binding protein-α (C/EBP-α) expression is decreased within the asthmatic ASM [71]. Since the promoter that regulates the expression of MLCK contains several C/EBP-α binding sites, such a deficit in C/EBP-α has been proposed to account for the increased expression of MLCK [72]. Nevertheless, such increased expression of MLCK in asthmatic ASM needs to be confirmed since controversial findings have also been reported [73, 74]. Regarding MLCK/MLCP-independent AHR, calcium-independent PKCε inhibits calponin, an actin thin filament-associated protein [75] that decreases calcium sensitivity [76]. It also activates mitogen-activated protein kinase (MAPK), which inhibits caldesmon, another actin

Table 3: Excitation/contraction coupling in ASM cells.

MLCK/MLCP balance	Extracellular factors	Intracellular factors	Actions	Consequences	References
Dependent		CaMKII	I: MLCK	R	[84]
	β-adrenergic agonist	PKA	I: MLCK	R	[85]
	β-adrenergic agonist	PDE4D increase	I: PKA	C	[86]
		PKG	A: MLCP	R	[87]
	TNF-α (TNFSF2), IL-13	Rho-kinase	I: MLCP	C	[87]
	TNF-α (TNFSF2)	Arachidonic acid	A: Rho kinase	C	[88, 89]
	TNF-α (TNFSF2)	Arachidonic acid	I: MLCP	C	[88, 90]
		CPI-17	I: MLCP	C	[87]
		C/EBP-α decrease	I: MLCK	C	[72]
Independent		Caldesmon	I: Myosin ATPase activity	R	[91]
		Calponin	I: Myosin ATPase activity	R	[92]
		PKCε	I: Caldesmon	C	[75]
		PKCε	I: Calponin	C	[75]
	Derp1, LPS	MAPK	I: Caldesmon	C	[75, 93, 94]
	TNF-α (TNFSF2), IL-13	Rho-kinase	I: Calponin	C	[95]
		CaMKII	I: Calponin	C	[95]
		HSP phosphorylated	I: Contraction	R	[96, 97]

A: active; C: contracting; CaMKII: calcium/calmodulin-dependent protein kinase; CPI-17: 17-kDa PKC-potentiated inhibitory protein of PP1; C/EBPα: CCAAT/enhancer binding protein α; Derp1: house dust mite allergen; HSP: heat shock protein; I: inhibit; IL-13: interleukin-13; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; PDE4D: phosphodiesterase 4D, cAMP-specific; PKA: cAMP-dependent protein kinase A; PKCε: protein kinase C ε; PKG: cGMP-dependent protein kinase G; R: relaxing; PP1: protein phosphatase 1; TNF-α: tumor necrosis factor α.

thin filament-associated protein [75] that also decreases calcium sensitivity [76]. Alternatively, RhoA/Rho-kinase signalling, which is increased in experimental asthma, inactivates MLCP leading to MLCK/MLCP-dependent AHR [77], and also inhibits calponin leading to MLCK/MLCP-independent AHR. Such RhoA signalling can be reproduced experimentally, using either the proinflammatory cytokine TNF-α (TNFSF2), which activates RhoA [78], or IL-13, which increases its expression in murine ASM [79].

Finally, the alteration in the dynamic properties of ASM in asthma is still a matter of current debate. The maximal velocity of shortening in ASM from sensitized animals is significantly greater than those in nonsensitized [80] and may be a consequence of an increase in the activity of MLCK [81] but is still controversial (see above). With respect to force generation, although isometric force generated *in vitro* is directly related to ASM mass in human bronchial ring preparations [82], force generation from asthmatic ASM was not found consistently increased [83].

2.4. Alteration in Contraction/Load Coupling. Like any muscle, ASM may overcome loads that normally moderate ASM shortening [98]. Two main categories of mechanical loads thus apply to ASM: a preload, which is directly related to lung elastic recoil and lung volume, and an afterload, which is determined by the shear modulus of the parenchyma and the coupling of the lung to the airways [98]. Some of these loads reside at the airway cellular/tissular level. Any alteration in such loads against which the ASM contracts and/or in the application of these loads to ASM will result in an alteration in shortening and, hence, airway narrowing.

In asthma, several mechanisms may account for an alteration in the contraction/load coupling [83, 98] such as an increase in lung elastic recoil pressure and a lung expansion that increase the external load that opposes ASM shortening. Indeed, loss of lung elastic recoil related to hyperinflation has been demonstrated in acute asthma [99] and chronic persistent asthma even without emphysema [100]. Moreover, a coupling between lung parenchyma and airways occurs through the attachments of elastic fibers to the airway wall [98]. In healthy individuals, periodic deeper inspirations (DIs) dilate the airways because of an excessive relative airway hysteresis as compared to parenchymal one [101]. In contrast, loss of the beneficial effect of DI is sometimes considered as a hallmark of asthma [102, 103]. Why DI induces bronchoconstriction in some asthmatic patients remains a matter of debate. Leading hypotheses put forward include neurohormonal mechanisms such as increase in cholinergic tone [104] or mechanical mechanisms such as calcium-dependent myogenic response of ASM [105] or loss of interdependence between lung parenchyma and airways [106]. Such uncoupling of the forces of interdependence may also be the result of airway inflammation and remodelling, especially thickening and/or oedema of the adventitia [107–109]. Moreover, stiffness of the airway wall may reduce the ability of tidal breathing and DI to stretch ASM, leading to latch bridges between actin/myosin and to a vicious positive feedback [110, 111]. Finally, altered organization and degradation of ECM proteins might also be less effective to moderate ASM shortening [83, 112, 113].

On the other hand, thickening of the airway wall and the enhanced amount of ECM also increase airway stiffness and

Table 4: Mediators secreted and immunomodulatory proteins expressed by human ASM cells.

Factors	References
Cell adhesion/costimulatory molecules, receptors	
CD11a	[117]
CD40 (TNFRSF5), CD40L (TNFSF5), CD44	[118]
CD80, CD86	[117]
OX40L (TNFSF4)	[119]
Adhesion molecules: ICAM-1, VCAM-1	[118]
Major histocompatibility complex (MHC) II	[120]
Toll-like receptors: TLR2, 3, 4	[121]
Chemokine receptors: CCR3, 7	[122]
Chemokine receptors: CXCR1, 3, 4	[122]
Receptors for IL-4, 6, 12, 13, 17, 22 and IFN-γ	[116, 123]
Chemokines, cytokines, and growth factors	
CCL2, 5, 7, 8, 11, 17, 19	[124–128]
CXCL8, 10	[129, 130]
CX_3CL1	[55]
IL-2, 5, 6, 11, 12	[57, 131]
Interferon- (IFN-) γ	[57]
Connective tissue growth factor (CTGF)	[132]
Granulocyte macrophage-colony stimulating factor (GM-CSF)	[133]
Stem cell factor (SCF)	[134]
Transforming growth factor- (TGF-) β1	[135]
Vascular endothelial growth factor (VEGF)	[136]
ECM proteins	
Chondroitin sulfate	[137]
Collagens I, III, IV, V	[137]
Decorin, elastin, fibronectin, laminin, perlecan	[137]
Thrombospondin	[137]
Tissue inhibitor of MMPs- (TIMPs-) 1, 2	[138]
Enzymes	
Matrix-metalloproteinases- (MMPs-) 9, 12	[139]

decrease airway compliance [114] that might enable the airways to resist to dynamic compression. Moreover, deposition of connective tissue also acts as mechanical impedance to contraction [113]. Nevertheless, these theoretical advantages of airway remodelling are largely overwhelmed by their negative effects [83].

3. Role of ASM in Bronchial Inflammation

While early studies regarding ASM function in asthma focussed on its contractile properties, a growing body of evidence now consistently demonstrates that ASM is no longer a sole target of the inflammatory process. Indeed, ASM cell also displays proinflammatory and immunomodulatory functions [115, 116], through its synthetic function and its expression of a wide range of cell surface molecules, integrins, costimulatory molecules, and Toll-like receptors (Table 4). As a consequence, ASM can play an active role, through an autocrine and/or a paracrine manner, in the asthmatic inflammatory process. The molecular and cellular mechanisms modulating ASM cell/inflammatory cell function will be reviewed below, with special attention to those implicating mast cells and T lymphocytes, which are known to infiltrate the asthmatic ASM layer [6].

3.1. ASM/Mast Cell Interaction. There is evidence that the asthmatic ASM layer is infiltrated by an increased number of mast cells [6, 55, 130, 140–147]. Some mast cells are already present in nonasthmatic ASM including smokers [148–152]. However, this mast-cell microlocalization within the asthmatic ASM, termed mast cell myositis [153], appears to be a specific feature of asthma, being absent in patients suffering from eosinophilic bronchitis and healthy subjects [140, 145]. Interestingly, the mast cell myositis is observed in various asthma phenotypes, including eosinophilic and noneosinophilic asthma [154], and also atopic and non atopic asthma, even if the number of mast cells is significantly higher in the ASM of atopic asthmatics [142]. Moreover, mast cell myositis is unchanged whether asthma is treated or not [145, 146], or regardless of asthma severity [143, 145–147].

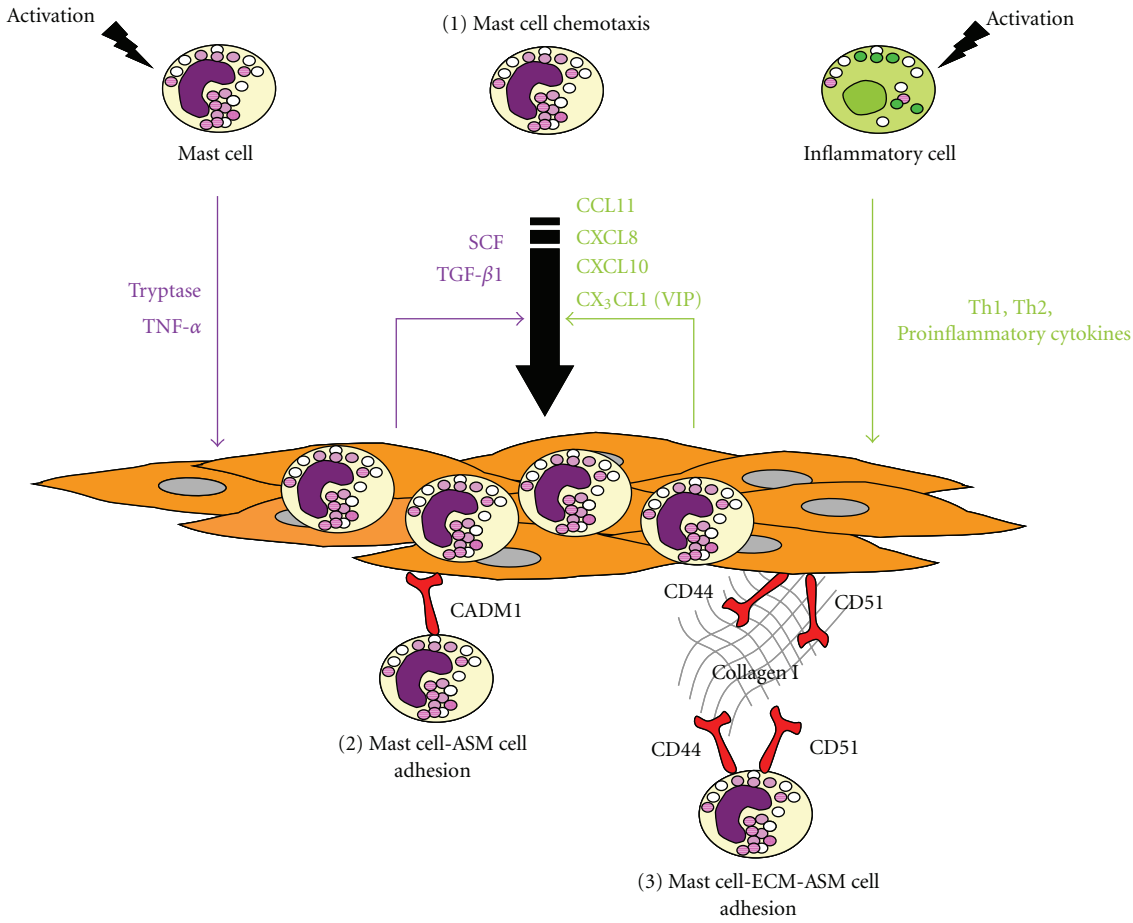


Figure 2: Mechanisms underlying mast cell myositis in asthma. In asthma, the mechanisms leading to an infiltration of airway smooth muscle (ASM) layer by mast cells, termed mast cell myositis, involve (1) mast cell chemotaxis towards the ASM bundle, (2) direct mast cell-ASM cell adhesion, and (3) mast cell-extracellular matrix- (ECM-) ASM cell adherence. Upon mast cell activation, mast cells release mediators which activate ASM cells, such as tryptase and TNF-α (TNFSF2). As a consequence, ASM cells produce and secrete chemotactic factors for mast cells, leading to an autoactivation loop. Under stimulation by Th1, Th2, and/or proinflammatory cytokines, produced by various inflammatory cells, ASM cells also secrete a wide range of mast cell chemotactic factors.

The mechanism of such a myositis has been firstly related to the production of mast cell chemotactic factors by the ASM itself, through an autoactivation loop [144] (Figure 2). Indeed, upon activation, mast cells release tryptase and proinflammatory cytokines, such as TNF-α (TNFSF2), which stimulate the production of TGF-β_1 and, to a lesser extent, SCF by ASM cells, which in turns, induce mast cell chemotaxis [144]. Moreover, ASM can promote mast cell chemotaxis through the secretion of a wide array of chemoattractants, upon stimulation by Th1 [130], Th2 [130, 155] or proinflammatory cytokines [55, 144]. For instance, ASM also produces functionally active CXCL10 [130], CXCL8 [155], CCL11 [155], and CX_3CL1 [55], even if, for CX_3CL1, the additional presence of VIP is necessary [55]. Taken together, these findings support the view that mast cell migration depends on various mediators secreted by ASM and is closely related to ASM inflammatory microenvironment.

Once present within the ASM bundle, mast cells can adhere to ASM. This adhesion has been initially ascribed to a cell-cell interaction involving an Ig superfamily member, that is, cell adhesion molecule 1 (CADM1), previously known as tumor suppressor in lung cancer 1 (TLSC-1) [156, 157] (Figure 2). However, blocking CADM1 leads to only a partial reduction in the adhesion of mast cells to ASM, suggesting additional mechanisms are present [156]. Indeed, mast cell-ASM adherence also involves cell-ECM-cell interaction through type I collagen, CD44, and CD51 [2] (Figure 2). This adhesion is improved under inflammatory conditions or using asthmatic ASM cells [2]. These *in vitro* findings are in agreement with ultrastructural analysis of asthmatic ASM using electron microscopy, demonstrating the absence of direct cell-cell contact between ASM and mast cells [6].

The majority of mast cells infiltrating the asthmatic ASM layer are typically of the MC_{TC} phenotype, containing both tryptase and chymase [140, 158, 159]. Interestingly, these mast cells infiltrate ASM in both large and small airways, and exhibit marked features of chronic ongoing activation [142, 143]. Such findings were also confirmed by ultrastructural analysis of ASM using electron microscopy [6]. However, little is known about the mechanisms by which mast cell activation may occur within the ASM layer [160]. Mast cell degranulation may result from IgE-dependent

activation, especially in atopic patients [142]. However, IgE-independent mechanisms have also been evoked, following mast cell-ASM interaction through the complement C3a or SCF [42, 157, 161, 162], for instance, or following bacterial or viral infection through Toll-like receptors [160].

Taking into account the following characteristics: (i) microlocalization of mast cells within the ASM layer, (ii) mast cell adherence to the ASM, and (iii) mast cell activation within the ASM, it is tempting to consider that a close functional relationship may exist between these two cell types.

On the one hand, mast cells alter functional and phenotypic properties of ASM cells. Indeed, mast cell-derived mediators contribute to AHR and ASM remodelling [12]. For instance, the major mast cell product, tryptase, induces both ASM calcium response [30] and AHR to histamine *in vitro* [60] or *in vivo* [163]. Tryptase also increases ASM cell-TGF-β1 secretion, which, in turns, promotes ASM cells differentiation towards a contractile phenotype, characterized by an increase expression of α-actin and enhanced contractility [42]. Interestingly, the number of mast cells within the ASM layer is positively correlated with the degree of AHR [140, 145], and with the intensity of α-smooth muscle actin [42].

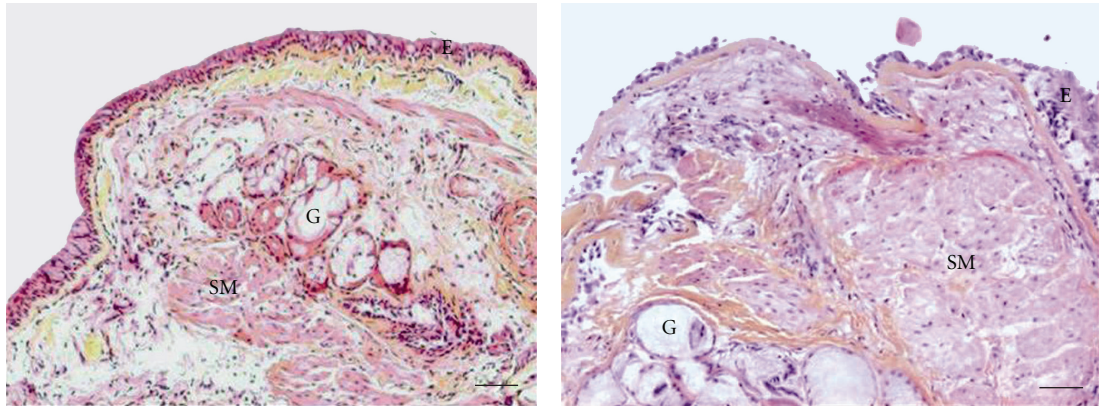
Mast cell myositis may also promote ASM remodelling. However, although several mast cell products such as tryptase are known to individually stimulate both DNA synthesis and ASM proliferation [164], coculturing ASM and mast cells does not increase proliferation [161]. Similarly, ASM survival is not enhanced by mast cell interaction [161]. Both mast and ASM cell-derived CCL19 mediate ASM migration through ASM CCR7 activation [128]. By contrast, CCL11/CCR3-mediated ASM cell migration was inhibited by mast cells [165]. No correlation was found between the number of mast cells in the ASM and ASM mass, supporting the modest role of mast cells in ASM remodelling [142].

On the other hand, ASM cells alter functional and phenotypic properties of mast cells. Indeed, ASM cells can promote mast cell survival, hence providing a favorable microenvironnement for mast cells [157]. They also enhance their proliferation, through a mechanism involving a cooperative interaction between ASM membrane-bound SCF, soluble IL-6, and mast cell-expressed CADM1 [157]. ASM cells-derived ECM proteins may also promote mast cells differentiation towards a fibroblastoid phenotype, characterized by the expression of fibroblast markers and fibroblast-like morphology. This feature seems to be specific of mast cells within the ASM layer, since fibroblast markers are not expressed in submucosal mast cells [147].

3.2. ASM/T Cell Interaction. Up to now, only few studies have documented T cell infiltration within the ASM layer in asthma. $CD4^+$ T cell microlocalization within ASM layer was first reported in an elegant experimental rat asthma model [166]. This finding was further confirmed in human asthmatics [6, 15] and is related to asthma severity [15]. Moreover, this feature appears to be specific of asthma, since T cells were not found in the ASM layer of control subjects, but within and beneath the epithelium [15]. As compared to mast cell ASM infiltration, there is a relative paucity of T cells within the ASM layer [6, 140]. However, ASM cells are able to produce appropriate chemotactic factors for T cells, such as CCL5 [125]. In this respect, a possible role for mast cell chymase has been suggested since this protease is known to inhibit T cell adhesion to nonasthmatic ASM cells *in vitro* [167]. Nonetheless, close contact were pointed out between ASM cells and T cells in asthma *ex vivo* [6, 15], hence suggesting cell-cell adherence between these two cell types.

Lazaar and coworkers originally demonstrated that activated T cells can adhere *in vitro* to resting ASM cells from nonasthmatic patients and that such an adhesion was enhanced when ASM cells were primed with proinflammatory cytokines such as TNF-α (TNFSF2) [118]. These findings were independently further confirmed [117]. This adhesion involves CD44, intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) expressed by ASM cells, and CD44, lymphocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4) expressed by T cells [118]. More recently, nonasthmatic ASM cells, pulsed to the superantigen staphylococcal enterotoxin A (SEA), have been shown to adhere to T cells by presenting the SEA *via* their MHC class II [168]. Although ASM cells express MHC class II, constitutively as well as under stimulation [117, 120], they are not classically considered as an antigen-presenting cell. Consequently, these findings support an emerging role of ASM cell as an immunomodulatory cell. However, except for VCAM-1, which forms clusters in the asthmatic ASM *ex vivo* suggesting VCAM-1 mediated intercellular signalling, the role of the above molecules in adherence between T cell and ASM cell from asthmatic patients has not yet been considered. Two other ASM cell-surface molecules, CD40 (TNFRSF5) [117, 169–171] and OX40 ligand (TNFSF4) [119, 171, 172], both expressed in asthmatic and nonasthmatic ASM cells, may also play a role in promoting ASM cell-T cell adherence. These costimulatory cell-surface molecules, members of the TNF superfamily, respectively, bind to CD40L (TNFSF5) and OX40 (TNFRSF4) on activated T cells [173, 174]. However, their role in the adherence of T cells to asthmatic ASM remains to be investigated.

Close interaction between T cells and ASM cells may lead to stimulatory cross-talk between these two cell types, but little is known about the functional consequences of such an interaction. On the one hand, T cells may alter functional properties of ASM cells. In this connection, T cells alter ASM contractile phenotype, enhancing ASM contractility to acetylcholine and reducing its relaxation to isoproterenol in isolated rabbit ASM tissue [117]. T cells may also drive ASM remodelling, in particular ASM hyperplasia [15, 118, 166]. Indeed, in an experimental rat asthma model, adoptively transferred $CD4^+$ T cells from OVA-sensitized rats increases ASM mass, which is both associated with an increased ASM proliferation and decreased apoptosis *ex vivo* [166]. Such an increased ASM proliferation and decreased apoptosis was confirmed *in vitro* only upon direct $CD4^+$ T cells-ASM cells contact, highlighting the need for close cellular interaction between these two cell types [166]. These observations are in agreement with a previous study



(a) (b)

Figure 3: Representative optic microscopic from bronchial sections stained with Haematoxylin, Eosin, and safranin stain were obtained from (a) a control subject or (b) an asthmatic patient (printed from Bara et al. [12], with permission of European Respiratory Journal publisher) E: epithelium; G: mucous gland; SM: smooth muscle. Scale bars represent 50 μm.

demonstrating *in vitro* a role for T cell in ASM DNA synthesis and proliferation in ASM cells from nonasthmatic patients [118]. The role of T cells in driving ASM remodelling was later confirmed in human asthmatics [15]. Moreover, the number of T cells infiltrating the asthmatic ASM correlates with ASM mass [15]. Collectively, these findings suggest an emerging role of T cells in both ASM hyperresponsiveness and remodelling.

On the other hand, ASM cells may also alter functional properties of T cells. Indeed, a direct contact between $CD4^+$ T cells and ASM cells also enhances T cell survival, thus possibly contributing to the perpetuation of bronchial inflammation [166]. Moreover, cultured human nonasthmatic ASM cells are able to present superantigens *via* their MHC class II molecules to resting $CD4^+$ T cells [168], which leads to $CD4^+$ T cells activation, adherence between these cells, and finally release of IL-13, that, in turns, leads to increase in the contractile response to acetylcholine of isolated rabbit ASM tissues [168].

4. Role of ASM in Bronchial Remodelling

ASM remodelling represents a key feature of asthmatic bronchial remodelling [12] (Figure 3). Indeed, many lines of evidence demonstrate an increase in ASM mass in fatal [175] and nonfatal asthma [15, 61, 70, 142]. However, ASM remodelling is still insensitive to current asthma medications [176] that are usually effective in treating acute airway narrowing and bronchial inflammation. Furthermore, ASM cell plays also a role in promoting other bronchial remodelling structural alterations, in particular by virtue of its synthetic function. For instance, ASM cells can release biologically active TGF-β [177], which is involved in various structural alterations such as epithelial changes, subepithelial fibrosis, mucus hypersecretion, goblet cell hyperplasia, and angiogenesis [178].

The mechanisms underlying ASM remodelling involve ASM hyperplasia, ASM hypertrophy, and an excessive deposition of proteins of the ECM [12].

4.1. ASM Hyperplasia. Three mechanisms may account for ASM hyperplasia, that is, (i) ASM cell proliferation, (ii) reduced ASM cell apoptosis, and more recently (iii) migration of myofibroblasts within the ASM layer.

4.1.1. ASM Cell Proliferation. Compelling evidence now suggests an excessive ASM cell proliferation in asthma both *in vitro* [61, 62, 71, 179, 180] and *in vivo* [15, 181]. However, before these two recent studies [15, 181], the number of proliferative ASM cells *in vivo* was a matter of debate since the number of PCNA or Ki67 positive ASM cells was unchanged in asthmatic bronchial samples [6, 70]. The reason for this discrepancy is apparently related with the fixation process.

A wide array of mitogenic factors are known to promote ASM cell proliferation *in vitro*, including growth factors, cytokines, chemokines, inflammatory mediators, enzymes, ECM components, reactive oxygen species, and mechanical stress (Table 5). Such mitogenic factors promote ASM cell proliferation by activating pathways involving either receptor tyrosine kinase (RTK) or receptor coupled to heterotrimeric *G* proteins (GPCR) [182]. Upon activation of these receptors, downstream transduction pathways usually involve Ras, phosphatidylinositol 3-kinase (PI3K) or MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK), which in turns induce cyclin D1 expression [182].

However, asthmatic ASM cells can also proliferate faster *in vitro* than nonasthmatic ASM cells irrespective of extracellular stimuli [179], hence supporting the view that an intrinsic ASM abnormality does exist in asthma. In this respect, ASM tissue-specific decreased levels of C/EBPα were found in asthmatics and could explain enhanced proliferation [71], through downregulation of the cell-cycle inhibitor p21. In addition, steroids are less effective in asthmatic ASM cells, since C/EBPα expression is impaired [71]. An alternative, or complementary, ASM tissue-specific explanation for the increased asthmatic ASM cell proliferation is an alteration of ASM calcium homeostasis. In severe asthma, an abnormal extracellular calcium entry leads to subsequent activation of peroxisome proliferator-activated receptor γ

Table 5: Mitogenic factors for human ASM cells.

Classification	References
Growth factors/cytokines/chemokines	
Platelet-derived growth factor (PDGF)	[211]
Fibroblast growth factor (FGF)	[212]
Epidermal growth factor (EGF)	[213]
Tumor necrosis factor- (TNF-) α (TNFSF2)	[214]
Transforming growth factor- (TGF-) β1 (controversial)	[215, 216]
CCL3, 5, 11	[186]
CXCL8	[186]
Inflammatory mediators	
Histamine	[217]
Endothelin-1	[218]
Thromboxane A2	[219]
Sphingosine 1-phosphate	[220]
Cysteinyl leukotrienes (LTC4, LTD4, LTE4)	[221]
Enzymes	
Tryptase	[164]
Thrombin	[222]
Elastase	[223]
Matrix metalloproteinases	[224]
ECM components	
Fibronectin	[225]
Collagen I	[225]
Others	
Reactive oxygen species	[226]
Mechanical stress	[227]
Brain derived neurotrophic factor (BDNF)	[228]

coactivator-1α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (mtTFA) [61]. This transduction pathway results in an increased mitochondrial biogenesis leading to enhanced ASM proliferation [61] (Figure 1). Interestingly, such enhanced mitochondrial biogenesis was not observed in other cell types, such as endothelial or epithelial cells, highlighting the smooth muscle specificity of such findings [61]. More recently, ASM calcium homeostasis has also been shown to be impaired in nonsevere asthma, *via* a different mechanism [62]. Such mechanism implicates reduced SERCA2 expression in both native and cultured ASM cells, but origin of such defect in asthmatic ASM remains unknown [183] (Figure 1).

4.1.2. Reduced ASM Apoptosis. Another explanation is to consider that ASM hyperplasia may be due to an imbalance between ASM proliferation and death. However, little is known about processes that may counterbalance ASM proliferation, such as apoptosis. Indeed, by contrast to the substantial evidence supporting ASM proliferation, only few studies have investigated ASM cells survival signals, ASM cell spontaneous apoptosis, or their susceptibility to apoptosis in response to proapoptotic factors. Most of the current knowledge about apoptotic pathways in ASM originates from nonasthmatic ASM cells.

Cardiotrophin-1 [184], endothelin-1 [185], chemokines, such as CCL3, CCL5, CCL11, and CXCL8 [186], or some ECM components, including fibronectin, laminin, and collagens I and IV [187], inhibit nonasthmatic ASM cell apoptosis. ASM cells express *in vitro* the death receptors TNFR1 (TNFRSF1A), Fas (TNFRSF6), TRAILR1 (TNFRSF10A), TRAILR2 (TNFRSF10B) [188], and stimulation of ASM cells either with TNF-α (TNFSF2) [188, 189], soluble Fas ligand (TNFSF6) [188], Fas antibody [189], or TRAIL (TNFSF10) [188] induces cell apoptosis. Similarly, hydrogen peroxide-exposure decreases ASM cells survival and is prevented by overexpression of the small heat shock protein 27 (Hsp27), in part by upregulating glutathione levels [190]. ECM-degrading proteinase, such as neutrophil elastase, may also induce ASM cell apoptosis by triggering detachment from the ECM [191], which results in the withdrawal of survival signals usually provided by some ECM components [187]. Conversely, some ECM components, such as decorin, may also induce ASM cells apoptosis *in vitro* [192]. Some treatments are also able to induce nonasthmatic ASM cell apoptosis *in vitro*. Indeed, activation of peroxisome proliferator-activated receptor γ (PPARγ), which is expressed by ASM cells, by its synthetic ligand commonly used in diabetes treatment, may also induce apoptosis [193]. Moreover, simvastatin, an HMG-CoA reductase inhibitor, has recently been shown to trigger ASM cells apoptosis through the intrinsic apoptotic pathway involving p53, enhanced mitochondrial permeability, mitochondrial release of Smac and Omi, and inhibition of mitochondrial fission, which in turns leads to the activation of caspases 9, 7 and 3 [194]. However, the role of these different mediators and signalling pathways in asthmatic ASM cell survival remains unknown.

In asthma, the hypothesis of an imbalance between ASM proliferation and apoptosis is mainly supported by a rat model of T-cell driven remodelling [166]. Furthermore, an increased expression of TRAIL (TNFSF10) has also been shown *ex vivo* in asthmatic ASM following allergen challenge, suggesting that this cytokine may play a role in asthmatic ASM apoptosis [195]. Besides, active caspase 3 expression and double stranded DNA breaking were shown *ex vivo* in ASM, in severe asthma, and in case of fatal asthma, respectively [188]. Conversely, such findings were not reported in intermittent asthma [188] and Benayoun and coworkers failed to demonstrate active caspase 3 expression *ex vivo*, irrespective of asthma severity [196]. *In vitro*, two reports have also drawn negative results, showing no change in spontaneous apoptosis within asthmatic ASM cells [61, 161]. As a consequence, further studies remain to be undertaken to determine whether or not there is a change in asthmatic ASM cell apoptosis.

4.1.3. Migration of ASM Cells and Myofibroblasts. More recently, it has been suggested that migration of ASM precursor cells, either located within the bronchial wall or derived from peripheral blood fibroblast progenitors, towards the ASM bundles may also participate in ASM hyperplasia [197]. Cellular migration is characterized by significant cytoskeletal remodelling with apparition of filopodia and lamellipodia to

increase directed movement along a concentration gradient (chemotaxis) and/or nondirected movement (chemokinesis) [197].

Migration of myofibroblasts may participate in ASM hyperplasia, by differentiating to ASM-like cells [197]. Such an hypothesis was based on the microlocalization of myofibroblasts between ASM bundles [6], while an increased number of myofibroblasts has been demonstrated within the lamina reticularis, especially after allergen challenge [198], and within the lamina propria [15].

However, the origin of myofibroblasts remains a source of debate and several hypotheses have been put forward. First, myofibroblasts may come from resident fibroblasts that have differentiated [199] or from dedifferenciation of ASM cells themselves, which have migrated out of the ASM bundles towards the lumen [199]. Indeed, a wide range of mediators may promote *in vitro* human nonasthmatic ASM cell migration, such as cytokines, growth factors, chemokines, and some ECM components [197] (Table 6). The major transduction pathways so far identified for ASM cell migration involve PI3K, Rho-kinase, MAPK, p38, and phosphorylation of Hsp27 [197, 200]. Migration is also a strongly calcium-dependent process [62, 128]. Thus, enhanced cell spreading, which might reflect migration, was observed in asthmatic ASM cells lacking SERCA2, while knocking down SERCA2 using small interfering RNA in nonasthmatic ASM cells leads to earlier lamellipodia and enhanced cell spreading [62]. Whereas this first study suggests that asthmatic ASM cells might migrate more than nonasthmatic ASM cells [62], such findings require further investigations, as well as addressing directly ASM cell migration *in vivo*.

Myofibroblasts can also originate from epithelial cells undergoing phenotypic changes through epithelial-mesenchymal transition process [201–203]. Another hypothesis is that myofibroblasts come from the recruitment of circulating bone-marrow-derived progenitors, termed fibrocytes [204–208]. Fibrocytes may be identified by the coexpression of the stem cell marker CD34 and/or the panhematopoietic marker CD45 and collagen-1 [209]. In this setting, a growing body of evidence supports the concept of fibrocytes trafficking to the lung in asthma, both in mouse asthma models [204, 208] and in humans [204–207]. Indeed, fibrocyte localization has been found within the bronchial wall in asthma [204], in particular, within the ASM bundles irrespective of asthma severity [206], close to the basement membrane [207], in the lamina propria [206], or below the epithelium [204, 208]. Fibrocytes differentiate into myofibroblasts, as evidenced by the concomitant expression of fibrocyte markers and α-smooth muscle actin [204, 205]. Mechanisms underlying such fibrocyte chemotaxis to the lung are in part mediated by ASM secreted-PDGF [206], which also promotes fibrocyte differentiation to myofibroblast [205]. Besides, such chemotaxis might likewise involve the chemokine receptors CXCR4, CCR2, CCR3, CCR4, CCR5, CCR7, and the chemokines CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, and CCL13 [210]. Interestingly, circulating fibrocytes number is positively correlated to the slope of the yearly decline in FEV_1 [205], once again suggesting a role of fibrocytes in ASM remodelling.

Table 6: Promigratory factors for human ASM cells.

Factors	References
Growth factors/cytokines/chemokines	
FGF2, PDGF, TGF-α (TNFSF2), TGF-β	[229, 230]
IL-1β, IL-17A, IL-17F, IL-22	[123, 230]
CCL5, 11, 19	[128, 231, 232]
CXCL8	[231]
Leukotriene E4	[233]
ECM components	
Collagens I, III, V	[233]
Fibronectin, laminin	[233]
Enzymes	
Matrix metalloproteinase- (MMP-)3	[234]
Others	
Thrombin	[235]
Urokinase plaminogen activator	[236]

4.2. ASM Hypertrophy. Unlike ASM cell hyperplasia, evidence for ASM cell hypertrophy in asthma remains a matter of debate [6, 70, 74, 175]. In fact, increased ASM cell size has been reported in tissue specimens from intermittent [70], mild-to-moderate [70], severe [70], fatal [175], and atopic asthma [6]. However, conflicting findings were found in mild-to-moderate asthma [74]. As a consequence, it has been suggested that ASM cell hypertrophy might be a hallmark of severe asthma status, since it can discriminate patients with severe asthma from those with milder disease [70]. Interestingly, ASM cell hypertrophy was associated with an increased amount of MLCK, which is involved in AHR [70]. Nevertheless, whether ASM hypertrophy is a condition sufficient to induce AHR in asthma needs further investigations.

The underlying mechanisms of such ASM cell hypertrophy remain unknown in asthma. Cardiotrophin-1 [184], serum deprivation [237], TGF-β [238], and endothelin-1 [185] can drive ASM cell hypertrophy *in vitro*, and only in nonasthmatic ASM cells. More recently, the role of miRNA-26a, whose expression is increased by mechanical stretch, has been highlighted in ASM cell hypertrophy [239]. However, the role of these factors needs to be clarified in asthma, as well as the putative role of SERCA2, whose expression is known to be also reduced in cardiac hypertrophy [240].

Two distinct transduction pathways can lead to ASM cell hypertrophy [241]. Pathways can involve the mammalian target of rapamycin (mTOR), 4E-binding protein (4E-BP), the transcription factor eIF4E [242], and S6 kinase [243] or the inhibition of glycogen synthase kinase- (GSK-) 3β [244], for instance, by the serine/threonine kinase Akt [241]. However, involvement of such transduction pathways needs to be further investigated in asthmatic ASM cell hypertrophy.

4.3. Altered ECM within the ASM Layer. Alteration in ECM represents another feature of ASM remodelling [12]. ECM is increased in asthma [6], as a result of increased deposition

of ECM proteins by airway resident cells, such as epithelial cells, fibroblasts, myofibroblasts, and ASM cells (Table 4). Its composition is different from that of nonasthmatics [139]. Histological examination of asthmatic bronchial samples reveals enhanced deposition of ECM proteins in the bronchial wall, such as collagens I, III, V, fibronectin, tenascin, hyaluronan, versican, laminin, lumican, and biglycan [245, 246], while collagen IV and elastin are decreased [247]. However, conflicting findings have also been reported regarding, the amount of collagen III [248, 249], collagen IV [250] and elastin [251] in the asthmatic bronchial wall. Interestingly, ECM is also increased all around ASM cells regardless of asthma severity [6, 252]. Such increase may be due to decreased matrix metalloproteinases (MMPs), which degrade ECM proteins, and/or increased tissue inhibitors of MMPs (TIMPs). Thus, overexpression of TIMP-1 and TIMP-2 may account for low MMPs activity in asthma [138]. The profile of ECM proteins produced *in vitro* by asthmatic ASM cells is also different from that of nonasthmatic ASM cells, with more perlecan and collagen I, and less laminin-α1 and collagen IV [253].

Nevertheless, whether such alterations in ASM ECM products contribute to modulate composition of the overall bronchial ECM remains unknown. Conversely, increasing evidence supports the role of ECM in modulating several aspects of ASM function [254]. In fact, fibronectin and collagen I have been reported to promote ASM proliferation from nonasthmatic donors in a dose-dependant fashion (Table 5), whereas laminin inhibits growth [255]. Fibronectin, collagens I, IV, and laminin also provide strong survival signals for ASM cells and such an effect is mediated at least in part through the fibronectin receptor/$\alpha 5\beta 1$ integrin [187]. Moreover, ASM cell contact with membranes coated with ECM components, such as fibronectin, collagens III and V, has been shown to enhance ASM migration [233] (Table 6). Synthetic properties of ASM cells may also be modulated by ECM components, such as IL-1β-dependent ASM secretion of CCL5 and CCL11 [256]. More recently, ECM has been involved in triggering ASM maturation to the contractile phenotype upon serum deprivation [257].

5. Conclusion

ASM cell plays a pivotal position in the pathophysiology of asthma as (i) a main effector of AHR, (ii) a proinflammatory and immunomodulatory cell through its synthetic properties and its expression of a wide range of cell surface molecules, and (iii) a leading cell involving in bronchial remodelling. If AHR and bronchial inflammation respond fairly well to conventional therapy such as bronchodilators and anti-inflammatory drugs, ASM remodelling remains insensitive to these treatments [176]. Targeting the various functions of ASM represents therefore a challenge for future asthma treatments [5, 12, 176]. In particular, research needs to focus on ways to prevent and/or reverse ASM remodelling. In this connection, targeting mitochondrial biogenesis and/or developing proapoptotic strategies to induce ASM cell death may represent an attractive area of investigation. In this respect, development of noninvasive tools for quantifying ASM remodelling has received increasing attention in order to evaluate on a long-term basis the efficacy of such treat ments [258].

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Review Article

Is There a Regulatory Role of Immunoglobulins on Tissue Forming Cells Relevant in Chronic Inflammatory Lung Diseases?

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Epithelial cells, fibroblasts and smooth muscle cells together form and give structure to the airway wall. These three tissue forming cell types are structure giving elements and participate in the immune response to inhaled particles including allergens and dust. All three cell types actively contribute to the pathogenesis of chronic inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD). Tissue forming cells respond directly to allergens through activated immunoglobulins which then bind to their corresponding cell surface receptors. It was only recently reported that allergens and particles traffic through epithelial cells without modification and bind to the immunoglobulin receptors on the surface of sub-epithelial mesenchymal cells. In consequence, these cells secrete pro-inflammatory cytokines, thereby extending the local inflammation. Furthermore, activation of the immunoglobulin receptors can induce proliferation and tissue remodeling of the tissue forming cells. New studies using anti-IgE antibody therapy indicate that the inhibition of immunoglobulins reduces the response of tissue forming cells. The unmeasured questions are: (i) why do tissue forming cells express immunoglobulin receptors and (ii) do tissue forming cells process immunoglobulin receptor bound particles? The focus of this review is to provide an overview of the expression and function of various immunoglobulin receptors.

1. Chronic Inflammatory Lung Diseases

The most prominent chronic inflammatory diseases of the lung are asthma and chronic obstructive pulmonary disease (COPD). These two common diseases are a major burden for public health and affect over 500 million people worldwide (World Health Organization: WHO/NMH/CHP/CPM/05.4.). Other chronic inflammatory lung diseases are hypersensitivity pneumonitis which is caused by antigen exposure and lung fibrosis, the cause of which is unknown and therefore the pathology is widely uncertain [1–7]. To an unknown reason the prevalence of all chronic inflammatory lung diseases is on the rise especially in Asia [8, 9].

In Europe and the USA cigarette-smoke-induced COPD was death cause no. 4 in 2008 and with a further increasing prevalence it is expected to become death cause no. 3 within the next decade. COPD is characterized by chronic inflammation of the small airways, with similar pathologies known for asthma [10–12]. These include airway constriction, hyperplasia, and hypertrophy of mesenchymal cells, increased mucus production, tissue remodeling, and finally tissue degradation, emphysema [10, 11]. The latter pathology is regarded as a different disease by some investigators [10].

Asthma is the most frequent chronic lung inflammation in children and is the major cause of absence from school and work. Several studies indicated that the prevalence of asthma is increasing, especially in countries with an increasing urban life style [9, 13–15]. However, beside extensive investigations worldwide the link of urban lifestyle and asthma is not understood. Some studies suggest that countryside living and contact with animals of the mother during pregnancy and in early childhood may be protective [16, 17]. Therefore these studies indicate a central role of the innate immunity as well as of the adaptive immunity [18, 19].

However, the molecular or cell biological events that lead to the similar pathogenesis of chronic inflammatory lung diseases in distinct segments of the lung are not fully understood. Worse, there are no curative drugs available and only the symptoms can be controlled. Asthma symptoms can be reduced by inhaled glucocorticoids, long-acting β2-agonists,

muscarinic receptor antagonists, or by leukotriene inhibitors, phosphodiesterase inhibitors, or anti-IgE antibodies [20–24]. Even there are an increasing number of drug classes being approved for asthma therapy from which all reduce the inflammation, but do not show any effect on the extensive airway remodeling which is well documented in childhood asthma [25–27].

In COPD there are not many therapeutic options besides inhaled glucocorticoid and long-acting $\beta 2$-agonists, and their efficacy is low in most patients [12, 28]. The fact that remodeling of the airways persists after the environmental stimulus is gone, while inflammation depends on the presence of a stimulus, points out that the immune response may not always be the initiating factor for chronic inflammatory lung diseases [29, 30].

The current hypothesis is that chronic inflammatory lung diseases are causatively linked to an overreactive or out of control immune response to environmental factors [30, 31]. Asthma and other chronic inflammatory lung diseases can be induced by nonallergic factors such as cold air, humidity, exercise, or psychological stress [32–35]. Of course the immune system plays a major role in the pathogenesis of chronic inflammatory lung diseases, but there is evidence that it may not be the initiating factor.

New investigations in primates and humans suggest two major events that predispose an individual to develop chronic inflammatory lung diseases during life: (i) maternal exposure to environmental factors that reorganize the lungs maturation during pregnancy and (ii) exposure to such factors in the first six years of life [29–31, 36–40]. The mechanism how maternal behavior or exposure to risk factors modifies the embryonic lung development may involve innate immunity or immunoglobulin synthesis [41, 42]. Interestingly one of the earliest signs of the lung's increased susceptibility to develop a chronic inflammatory disease is the remodeling of the large or small airways [25, 36, 37]. Unfortunately, we do not understand the mechanisms how environmental factors increase the susceptibility of the lung to develop chronic inflammation upon a second, independent (unknown) triggering event. Neither do we understand the disease specific mechanism(s) that leads to the wide range of clinical phenotypes, nor for the different age of onset, nor the gender specificity [43–45]. In the past two decades we accumulated knowledge of the immune response in chronic inflammatory lung diseases, but this knowledge did not help us to explain the entire pathology of asthma or COPD or of any other chronic inflammatory lung disease.

2. The Immune Response of Tissue-Forming Cells

Tissue-forming cells have been excluded from studies of the immune response for a long time as it was assumed that these cell types do only respond to immunoglobulin indirectly through cytokines and growth factors which are released by activated immune cells after antigen or immunoglobulin binding. However, there is evidence that this is not the full story. Evidence for an active participation of tissue-forming cell in the lungs response to allergens is provided by a handful of research groups and thus the literature is rare. In allergic asthma and some forms of COPD the immune response is a central mechanism that initiates the pathology; however, we do not fully understand how it works. The immune response is a major cause of exacerbation in allergic asthma, but it cannot explain how nonallergic asthma is caused [32, 44, 46]. Asthma exacerbation can be induced by exercise, stress, humidity, cold or hot air [19, 46]. However, the immune response of the lung seems to be more complex than anticipated for the past decades. Besides immune cells the tissue-forming cells of the lung express immunoglobulin receptors and respond to immunoglobulins.

To understand the cause of chronic inflammatory lung diseases one has to include the contribution or the causative role of tissue-forming resident cell types which give the lung its structure and guarantee its function. In 1922 asthma was first described as a disease caused by overreactive airway smooth muscle cells and an increased size and number of smooth muscle bundles surrounding the airways [47]. Then the role of the immune cells and their response to environmental factors were regarded as being more important; however, recent studies refocused on the role of the smooth muscle and its interaction with other cell types [36, 48–52]. In COPD the disrupted interaction of epithelial cells with the submucosal fibroblasts has moved into the centre of attention in the past years [36, 53–56]. However, to identify the pathologic mechanism(s) that leads to chronic inflammatory lung diseases one has to understand the concerted interaction of all cell types with each other and this includes tissue-forming cells and immune cells (Figure 1).

One important aspect of allergic chronic inflammation is the response of the lung to inhaled allergens. Therefore it is surprising that the fact that tissue-forming cells include bronchial epithelial cells, fibroblasts, and airway smooth muscle cells has drawn not much attention, while the response of immune cells was such much in the focus during the past decades. First reports on immunoglobulin receptor expression and function in tissue-forming resident lung cells were published in the 1980s, but did not trigger extended investigations. In the following I will summarize the knowledge of immunoglobulin receptor expression on tissue-forming cells of the human lung and their possible contribution to the pathogenesis of mainly asthma and COPD.

3. Epithelial Cells

Epithelial cells of the airway form the barrier between the tissue and the inhaled air. They are the first cell type which is exposed to inhaled allergens, dust particles, vapor or chemicals. The epithelial cell layer consists of two subtypes, the outer ones are ciliated cells which are considered as end-differentiated cells and which are shed off when they do not function properly [57, 58]. The outer ciliated epithelial cells are followed by a layer of basal epithelial cells, which are a type of precursor and can transform into goblet cells

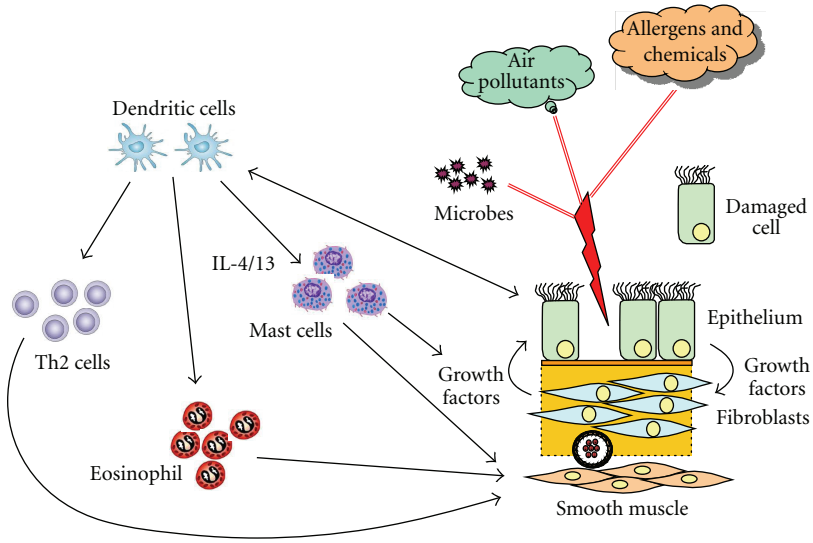

Figure 1: The network of interactions between tissue-forming resident airway cells and immune cells.

or ciliated epithelial cells [53, 58–60]. The basal epithelial cell layer is followed by the basement membrane which consists of pure extracellular matrix (ECM). It was assumed that the basal membrane forms an uninterrupted barrier between epithelial cells and subepithelial fibroblasts [45, 57]. However this view is challenged by several reports that indicate direct cell-cell contacts between epithelial cells and subepithelial fibroblasts [61–64]. If such direct cell-cell interactions between epithelial cells and submucosal fibroblasts exist, they must bridge the basal membrane and this may act as a direct passage of inhaled substances into the airway wall [65–68].

In asthma the number of columnar epithelial cells is shed off more frequently and the number of goblet cells is increased [59, 60]. Shedding may explain the reported increased epithelial cell fragility; however, to increase shedding additional factors must weaken the intercellular attachment of neighboring epithelial cells and the basal epithelial cells [57, 58, 61]. In COPD the direct epithelial cell-fibroblast interaction seems to be disrupted [36, 53–56]. It is also indicated that an abnormal expression or activity of adhesion molecules coupled with an alteration in the composition of the extracellular matrix (ECM) in asthma and COPD changes the response of the tissue-forming cells to antigens and Ig receptors [65, 67, 68].

Epithelial cells respond to inhaled particles and allergens not only through immune cell released cytokines, but also through cell membrane receptors [69–72]. Human bronchial epithelial cells express the low-affinity IgE receptor (CD23) as well as the high-affinity receptor and responded to its activation [73, 74]. The activation of the low-affinity IgE receptor resulted in secretion of endothelin-1, a well-known stimulator for fibrotic processes [74], and the activation of the high-affinity IgE receptor led to the release of 15-hydroxyeicosatetraenoic acid from epithelial cells of asthma patients only [73]. However, since this report by Campbell et al. no other study investigated the role and function of the low-affinity IgE receptor on epithelial cells. In epithelial cells of the intestine of allergic patients it had been shown that an allergen-IgE complex activated IL-8 secretion via the intracellular signal proteins Erk1/2 mitogen-activated protein kinase (MAPK) [75].

In an animal model it was suggested that allergen inhalation upregulates the expression of the polymeric Ig receptor by bronchial epithelial cells and its stimulation increased IgM and IgA secretion [76]. The study showed that this effect was paralleled by Th17 cell activation, but did not provide direct evidence for such a link. If such a mechanism could be confirmed in humans it would add significant weight to the regulation of immune response by tissue-forming cells.

In vitro experiments in a rat model suggest that alveolar epithelial cells also are able to express the IgG receptor and its expression is affected by glucocorticoids [77]. In other species and epithelial cells of other organs than the lung it had also been reported that the IgG receptors are expressed and are functional [78, 79]. Importantly it was also shown that the predisposition to allergies can be mediated by breastfeeding through maternal IgG and its receptor expression on embryonic lung epithelial cells [80]. One study performed in rat epithelial cell monolayers suggested that IgG via the Fc receptor enables antigen to be transferred through the epithelium or the epithelial cell, respectively, unchanged and be secreted on the apical side to subepithelial mesenchymal cells [77]. This would be an important new mechanism which will change the thinking of allergen presentation and immune response in the lung if it could be proven in an animal model or in humans.

Together these observations may be helpful to understand particle trafficking through the epithelium barrier in the lung and the contact of submucosal mesenchymal cells to such environmental factors [77, 81, 82]. The suggested

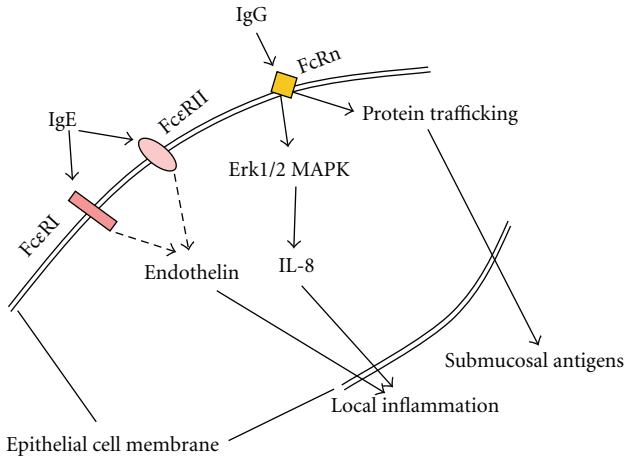

Figure 2: Epithelial cells express IgE and IgG receptors and respond directly to the respective immunoglobulins. Suggested (dashed line) and proven intracellular signaling pathways in human and animal airway epithelial cells. Importantly the IgG receptor expressed on airway epithelial cells may enable antigens to path un-changed through the epithelium and then contact with subepithelial mesenchymal cells [77].

functions of immunoglobulin receptors on epithelial cells are summarized in Figure 2. Unfortunately no studies have confirmed these data in humans.

4. Airway Fibroblasts and Fibrocytes

Bronchial submucosal fibroblasts have been also reported to be involved in the response to environmental factors and to viral infection. The innate immune system was activated by rhinovirus infection in humans and induced interferon-g synthesis. Furthermore, it was shown that the virus reproduced in submucosal human bronchial fibroblasts [83]. In an ovalbumin inducible airway inflammation mouse model it was reported that submucosal fibrogenesis was induced by the allergen through a Smad3-dependent pathway; however the precise mechanism how the allergen activated the fibroblast type cells was not characterized [84]. Another study indicated that the increased submucosal airway remodeling upon allergen stimulation involves the recomposition of the ECM and is directed by the communication of epithelial cells and submucosal fibroblasts [85].

While no study reported the expression and function of any immunoglobulin receptor on lung fibroblasts there is indirect evidence that this cell type must express such receptors. Knight et al. reported in 1999 that IgE as well as IL-1β stimulation increased leukemia inhibitory factor (LIF) and its receptor (LIFR) in human lung tissue, with the highest expression in fibroblasts [86]. However, since these experiments were performed in isolated tissue sections it might be that the response of the fibroblasts involved their activation by mast cells.

Fibrocytes that were differentiated from $CD14^+$ blood cells and exposure to serum amyloid P, which bound to the IgG receptor inhibited the signalling leading to fibrocyte differentiation. Furthermore, monoclonal antibodies binding to IgG receptor I (CD64) or II (CD32) also inhibit fibrocyte differentiation, indicating that tissue structure can be directly modified by immunoglobulins [87].

5. Airway Smooth Muscle Cells

Bronchial or airway smooth muscle cells belong to the best studied tissue-forming resident cells in asthma and COPD. As mentioned above asthma was first be considered as a disease of the airway smooth muscle [47]. Most if not all asthma patients show a significant increase of airway smooth muscle bundles and cell numbers within the bundles [25, 36, 50]. Investigations in childhood asthma and a more recent asthma model in rhesus monkeys suggest that tissue remodeling occurs already during pregnancy and is further increased during the first 6 years of life: mostly before any sign of inflammation can be found [25, 36, 50, 88–92]. Other studies even suggest that the exposure to environmental factors during pregnancy starts a mechanism that deregulates the lung maturation and predisposes the embryo to develop chronic inflammatory lung diseases later in life [26, 36–41, 92, 93].

In regard to COPD the exposure to cigarette smoke seems to be an essential trigger; however, what sets the lung to develop a chronic inflammation as a response to allergic asthma stimuli is not known [39, 40, 44, 94, 95]. In asthma models in rhesus monkeys the significant structural change of the airway smooth muscle cell bundles around the airways of house dust mite and ozone-challenged animal is impressive [92]. This observation is that in asthma the smooth muscle cell bundles arrange in a spiral-like structure which constricts the airways much more forcefully than the randomly arranged muscle bundles in a healthy airway [92]. This leads back to some questions from the late Professor A. Woolcock (Sydney University Royal Prince Alfred Hospital, Sydney, Australia) to her students. What is the function of the smooth muscle bundles in the normal lung? Why do we need smooth muscle bundles around the airways? These questions have never been answered.

Among the new classes of asthma therapies are antibodies that bind to IgE and neutralize it [24]. Anti-IgE antibodies bind to the IgE receptor docking site and thus prevent binding of IgE to its receptors. Recent studies indicate that this new class of asthma drugs seems to be very effective in asthma therapy and significantly reduces symptoms and the need of other medications [96, 97]. It would be too easy to argue that the beneficial effect of the anti-IgE antibodies is achieved by the disruption of the IgE-IgE receptor interaction of immune cells.

There are several studies starting from the late 1990s that provide evidence that the airway smooth muscle cell expresses and responds to the low as well as to the high IgE affinity receptors [98–103]. Furthermore, there is evidence that the expression of the IgE receptors is increased in asthma [102, 103]. Inhibition of IgE significantly reduced the secretion of proinflammatory cytokines by airway smooth muscle cells, and it also reduced their proliferation, at least

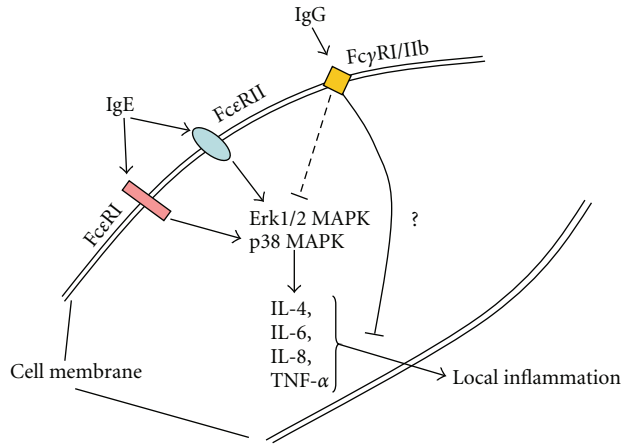

Figure 3: Expression and function of immunoglobulins and their receptors on human airway smooth muscle cells. Likely interaction and crosstalk of Ig receptors through shared intracellular signaling pathways. Reported pathways by which Ig receptors modulate cytokine synthesis by tissue-forming cells in chronic inflammatory lung diseases.

in vitro [104–106]. Furthermore, it has recently been shown that IgE activates the transcription and expression of the thymic stromal lymphopoietin in human airway smooth muscle cells, a factor which is important to the recruitment of circulating inflammatory cells into the lung [107]. This finding supports the idea that the tissue-forming cells of the airway wall significantly contribute to the immune response and that they may be the first cells in the line of defense, which are; however, deregulated in chronic inflammatory lung diseases.

In response to IgE stimulation airway smooth muscle cells have been shown to secrete proinflammatory cytokines including IL-8, and eotaxin, which will attract neutrophils and granulocytes to infiltrate the airway wall and thereby extend the local inflammation [99, 101, 108]. These observations are in line with clinical studies showing an anti-inflammatory effect of anti-IgE antibodies. In contrast Xia et al. [109] did not find the expression of any IgE receptor on tissue-forming cells in tissue sections. This discrepancy to other studies [98–103] may be explained by different methods or antibodies to detect IgE receptors. Further studies have to reassess the tissue compartmental expression of Ig receptors in the healthy and diseased lung.

Recently it was reported that human airway smooth muscle cells also express the IgG receptors, FcγRs-I, and -IIb [109]. Furthermore, the activation by IgG downregulated IL-1α induced cytokine production and reduced the activation of the two signaling proteins Erk1/2 MAPK and p38 MAPK. In sharp contrast to other studies the authors did not find any expression of the IgE receptors in their isolated cells. No reports are available for the expression of other immunoglobulin receptors on human airway smooth muscle cells. The possible function of the immunoglobulin receptors on human airway smooth muscle cells is summarized in Figure 3.

The above scarce data is evidence that the role of tissue-forming resident cell activation by immunoglobulins, not only IgE, must be investigated in more detail.

6. Conclusion

Tissue-forming cells of the human lung express and respond to the activation of at least two immunoglobulin receptors, IgG and IgE receptors. Thereby they directly contribute directly to inhale environmental factor such as allergens or dust without the need to activate immune reactive cells. The mechanism(s) that induce and control the expression of immunoglobulin receptor in these cell types have not been studied extensively. The relevance of the *in vitro* data on immunoglobulin receptor expression by tissue-forming resident lung cells has to be confirmed *in vivo* and its relevance to the pathologies of chronic inflammatory lung diseases has to be demonstrated. Ignoring the fact that these cells are an active participant in the lung's immune response to inhaled environmental factors will only delay the search for a better understanding of the distinct pathologies of the different chronic inflammatory lung diseases, and it will delay the search for new therapeutic strategies.

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Research Article

Effects of β_2 Agonists, Corticosteroids, and Novel Therapies on Rhinovirus-Induced Cytokine Release and Rhinovirus Replication in Primary Airway Fibroblasts

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Rhinovirus-(RV-) induced asthma exacerbations account for high asthma-related health costs and morbidity in Australia. The cellular mechanism underlying this pathology is likely the result of RV-induced nuclear-factor-kappa-B-(NF-κB-) dependent inflammation. NF-κB may also be important in RV replication as inhibition of NF-κB inhibits replication of other viruses such as human immunodeficiency virus and cytomegalovirus. To establish the role of NF-κB inhibitors in RV-induced IL- 6 and IL-8 and RV replication, we used pharmacological inhibitors of NF-κB, and steroids and/or β_2 agonists were used for comparison. Primary human lung fibroblasts were infected with RV-16 in the presence of NF-κB inhibitors: BAY-117085 and dimethyl fumarate; β_2 agonist: salmeterol; and/or corticosteroids: dexamethasone; fluticasone. RV-induced IL-6 and IL-8 and RV replication were assessed using ELISAs and virus titration assays. RV replicated and increased IL-6 and IL-8 release. Salmeterol increased, while dexamethasone and fluticasone decreased RV-induced IL-6 and IL-8 ($P < 0.05$). The NF-κB inhibitor BAY-117085 inhibited only RV-induced IL-6 ($P < 0.05$) and dimethyl fumarate did not alter RV-induced IL-6 and IL-8. Dimethylfumarate increased RV replication whilst other drugs did not alter RV replication. These data suggest that inhibition of NF-κB alone is unlikely to be an effective treatment compared to current asthma therapeutics.

1. Introduction

Asthma is a chronic inflammatory disease of the airways characterised by reversible airflow obstruction, inflammation, and hyperresponsiveness to various allergic or non-allergic stimuli such as house dust mites or exercise [1].

An asthma exacerbation is the increase in the duration and severity of respiratory symptoms often resulting in hospitalization. Respiratory viruses cause 85% of asthma exacerbations, and 62% of all viral induced asthma exacerbations are caused by human rhinovirus (RV) [2]. Viral-induced asthma exacerbations account for over 50% of the total asthma-related health costs and also increase asthma morbidity.

The bronchial epithelium has always been considered as the primary site of RV infection; however, increasing *in vivo* evidence shows that RV can also infect submucosal cells such as fibroblasts and airway smooth muscle (ASM) [3, 4]. *In vitro* studies have shown that transformed and primary human airway cells infected with RV release a plethora of proinflammatory cytokines, such as interleukin (IL)-6, IL-8, and the antiviral cytokine: interferon (IFN)-λs [3, 5–7]. The mechanism of this is most likely due to RV-activated nuclear factor kappa B (NF-κB).

NF-κB is a transcription factor, implicated in the expression of over 100 proinflammatory genes which mostly participate in the host immune response [2, 8]. NF-κB is exploited by many viruses such as RV and retroviruses to

promote their replication by preventing viral-induced apoptosis and evading the immune system [8, 9].

Previous studies have found that RV can activate NF-κB via IκB kinase (IKK)-α/β or phosphorylation of IκB and cause the upregulation of numerous cytokines [2, 10, 11].

Despite the vast array of asthma medication available, β_2 agonists and corticosteroids remain the most effective treatments in asthma to control and prevent symptoms [12]. However their use does not prevent asthmatics from respiratory viral infections or RV-induced asthma exacerbation [13]. Therefore there is a need for specific treatment strategies for RV-induced asthma exacerbation.

Studies have shown that inhibition of NF-κB reduces viral-induced cytokine release and also inhibits replication of human immunodeficiency virus (HIV) and human cytomegalovirus (HCMV) [14, 15]. However to date no research has examined the effect of inhibiting NF-κB on RV-induced cytokine release and RV replication in airway cells. For this reason, this study investigated if NF-κB inhibitors, either alone or in combination with the currently used asthmatic drugs, β_2 agonists and corticosteroids, reduce both RV-induced cytokine release and RV replication in human primary airway fibroblasts. This study may provide *in vitro* data that may be beneficial in determining a more adequate treatment regimen for RV-induced asthma exacerbations.

2. Materials and Methods

2.1. Isolation and Culture of Human Fibroblasts. Primary human airway fibroblasts were obtained from macroscopically healthy lung tissue. Lung tissue was obtained from patients undergoing resections or transplantations (see Table 1 for demographics).

Parenchymal tissue was washed in sterile Hanks balanced salt solution (Trace Scientific, Melbourne, Australia), minced and suspended in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Castle Hill, Australia) supplemented with 10% (v/v) foetal bovine serum (FBS) (JRH Biosciences, Melbourne, Australia), 20 U/mL penicillin, 20 g/mL streptomycin, and 2.5 g/mL amphotericin B (Invitrogen, Mount Waverley, Australia) in 75 cm^2 tissue culture flasks. The cells were grown to confluence, and fibroblast characteristics were confirmed by normal fibroblast growth patterns and cell morphology as described previously by Ghildyal et al. [7]. All experiments were carried out with fibroblasts between passages 2 and 8.

Ethical approval for all experiments involving the use of human lung tissue was provided by The University of Sydney Human Ethics Committee and the Sydney South West Area Health Service, and written informed consent was obtained.

2.2. RV Propagation and Ultraviolet Inactivation of RV (UVi-RV). Major group human RV serotype-16 was purchased from ATCC (Manassas, USA) and propagated in Ohio HeLa cells as previously described by Papi and Johnston [16]. In some experiments RV was UV inactivated in 24 well plates containing 200 μL of RV/well at a distance of 5 cm from a 30 W UV light source (germicidal lamp G30T8, Sankyo Denki, Japan) for 15 minutes. UV inactivation was established to be effective by a RV titration assay and was used as a noninfectious virus control.

Table 1: Demographics of donors from whom fibroblasts used in this study were isolated.

Patient	Disease	Sex	Age (years)
1	Transposition of the great arteries	M	39
2	Chronic obstructive pulmonary disease (COPD)	M	56
3	No disease	M	48
4	Idiopathic pulmonary fibrosis (IPF)	M	61
5	Emphysema	M	63
6	Small cell carcinoma	M	63
7	Lymphangioleiomyomatosis (LAM)	F	51
8	Pulmonary fibrosis	M	53
9	Bronchiectasis	M	53
10	Emphysema	M	40
11	Non-small cell carcinoma	M	77
12	Cystic Fibrosis	M	45
13	Non Small Cell Carcinoma	F	63
14	Non-small cell carcinoma	F	79
15	Primary pulmonary hypertension	F	36
16	Lymphangioleiomyomatosis (LAM)	F	33
17	Emphysema	F	56
18	Emphysema	F	48
19	Melanoma	M	63
20	Carcinoma	M	59
21	Lesion	F	58
22	Resection	M	48
23	Carcinoma	F	83
24	Idiopathic pulmonary fibrosis (IPF)	M	57
25	Carcinoma	F	76
26	Emphysema	F	50
27	Idiopathic pulmonary fibrosis (IPF)	M	56
28	Pulmonary fibrosis	M	68
29	Rejection:pneumonitis	M	21
30	α1 antitrypsin deficiency	M	55
31	Hypersensitive pneumonitis	M	59
32	α1 antitrypsin deficiency	M	42
33	Emphysema	M	42
34	Bronchiectasis	M	39
35	Small cell Carcinoma	F	78

2.3. Drug Concentrations. Dexamethasone, BAY-117085 (BAY), dimethyl fumarate (DMF) (Sigma-Aldrich), fluticasone propionate, and salmeterol (GSK, Boronia, Australia) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at 10^{-3} M and further diluted in 0.1% FBS/antibiotics/DMEM to give final experimental concentration

ranges of 10^{-12}–10^{-6} M and corresponding vehicle controls 0.001–0.1% DMSO. For drug combination experiments, the lowest concentration of drug to produce an effect was used.

2.4. RV Infection of Primary Human Airway Fibroblasts. Fibroblasts were seeded at 3.2×10^4 cells/mL into 6 well plates in 10% FBS/DMEM and grown for 3 days. Prior to RV infection, a cell count was carried out to determine the amount of RV (or UVi-RV) needed to infect at a multiplicity of infection (MOI) of 0.1. The medium was then replaced with 0.1% FBS/antibiotics/DMEM and or drug/vehicle and incubated for 1 hour at 37°C and 5% CO_2. Some of the wells were infected at an MOI of 0.1 with UVi-RV or live RV respectively and left for 1 hour at 37°C and 5% CO_2. Plates were rocked every 15 minutes to disperse the virus or contents. The medium was removed, the cells were washed with the Hanks solution, and 2 mL/well of fresh sterile 0.1% FBS/antibiotics/DMEM or drug/vehicle was added. The plates were then incubated at 37°C and 5% CO_2 over time, and supernatants were collected and stored at −20°C prior to analysis using ELISA and RV titration assays.

2.5. RV Titration Assay. All viral concentrations were measured by a titration assay as outlined by Papi and Johnston [16]. Briefly, RV levels were determined by serially titrating log-diluted concentrations of the cell-free supernatant in quadruplicates on the Ohio HeLa cells. The Ohio HeLa cells were seeded at a concentration of 2×10^4 cells/mL in 96 well plates (150 μL/well) and then 50 μL of supernatants with RV or control medium was added to the wells. The plates were rocked at 100 rpm for 15 minutes at room temperature before being cultured for 5 days at 37°C and 5% CO_2. After 5 days of culture, the cytopathic effect (CPE) was assessed by comparing the cells in the RV-infected wells to the control wells. Viral concentration was determined using Karber's method as described previously [7]. The concentration of RV-16 stock was determined to be 6.3×10^5 virions/mL.

2.6. ELISA. ELISA kits for IL-28A (interferon λ_2), IL-29 (interferon λ_1), IL-6, and IL-8 were purchased from R&D Systems (Minneapolis, USA) and BD Biosciences (North Ryde, Australia), respectively. ELISAs were carried out according to the manufacturer's instructions. The detection limits of these assays were 62.5 pg/mL (IL-28A), 31.2 pg/mL (IL-29), 7.8 pg/mL (IL-6), and 15.6 pg/mL (IL-8).

2.7. Statistical Analysis. Since there were no differential responses to RV in cells from patients with different diagnoses, results were pooled and analysed together in this study. All data were verified for normality and values presented as mean ± SEM. When results were nonparametrically distributed, the dataset was log transformed prior to statistical analysis using GraphPad Prism Version 5 software (Calif, USA). ELISA and RV titration results were analysed by either a 1-or 2-way analysis of variance (ANOVA) with the Dunnett or Bonferroni posttest comparisons where appropriate and indicated. Statistical significance was shown when $P \leq 0.05$.

3. Results

3.1. RV Infects Human Primary Airway Fibroblasts and Stimulates IL-6 and IL-8 Production but Not IL-28A (Interferon λ_2) and IL-29 (Interferon λ_1). To determine if RV induced IL-6 and IL-8 release and replicated in fibroblasts, tissue culture medium of infected fibroblasts was assessed using RV titration assay and ELISA at 0, 3, 6, 24, 48, and 72 hours post infection.

RV replicated in fibroblasts and was maximal 24h post infection when compared to 0 hours ($P < 0.0001$; $n = 5$; Figure 1(a)). There was no statistical significance between the number of virions at 24 and 48 hours post infection.

As can be seen in Figures 1(b) and 1(c), RV-induced IL-6 and IL-8 were maximal at 48 hours, compared to respective constitutive release ($n = 5$, $P < 0.0001$). No induction was observed with UVi-RV.

RV-16 did not induce IL-28A and IL-29 from human primary airway fibroblasts ($n = 5$, data not shown).

3.2. Corticosteroids Suppress and β_2 Agonists Increase Primary Airway Fibroblast Responses to RV Infection. To determine if the corticosteroids dexamethasone and fluticasone and the β_2 agonist salmeterol could inhibit RV-induced IL-6 and IL-8 and RV replication, tissue culture medium from fibroblasts pretreated with drug for 1 hour and then infected with RV was analysed by ELISA after 48 hours and RV titration 24 hours post infection.

As before RV induced IL-6 and IL-8 (Figure 2, $n = 7$–9, $P < 0.05$). Dexamethasone significantly inhibited both RV-induced IL-6 and IL-8 at concentrations greater than 10^{-10} M and 10^{-8} M, respectively (Figures 2(a) and 2(b), $n = 7$, $P < 0.05$). Fluticasone significantly inhibited both RV-induced cytokines at all concentrations tested 10^{-10}–10^{-8} M (Figures 2(c) and 2(d), $n = 7$, $P < 0.05$). Dexamethasone did not inhibit the constitutive release of IL-6 and IL-8 at the concentrations tested ($n = 7$, $P > 0.05$), while fluticasone inhibited the constitutive release of IL-6 and IL-8 at all concentrations (10^{-10}–10^{-8} M; $n = 7$, $P < 0.05$) (Table 2). However salmeterol further increased RV-induced IL-6 and IL-8, almost 2-fold more than RV control at concentrations 10^{-8} to 10^{-7} M (Figures 2(e) and 2(f), $n = 9$, $P < 0.05$). Salmeterol significantly induced the constitutive release of IL-6 at 10^{-8} M and IL-8 at 10^{-8} and 10^{-7} M, (Table 2, $n = 9$, $P < 0.05$). The highest concentration of vehicle used had no significant effect on the level of IL-6 and IL-8 induction. Dexamethasone, fluticasone, and salmeterol did not alter RV replication (data not shown).

3.3. NF-κB Inhibitors Increase RV Replication and Suppress RV-Induced IL-6 in Primary Airway Fibroblasts. To determine if the NF-κB inhibitors, BAY and DMF could inhibit RV-induced IL-6 and IL-8 and RV replication, tissue culture medium from fibroblasts pretreated with drugs and then

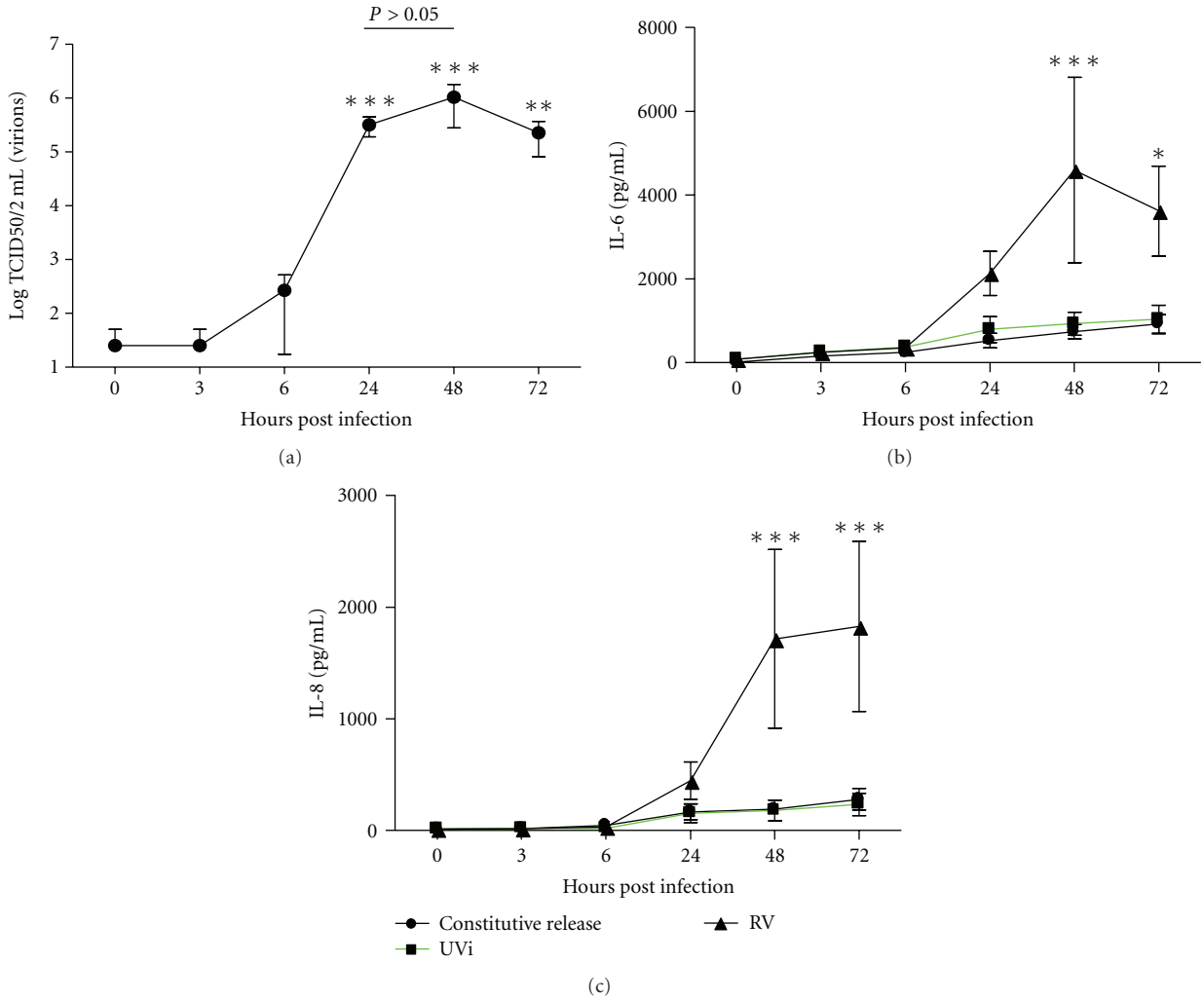

Figure 1: (a) Time course of RV replication. Concentration is of RV from infected fibroblasts (MOI = 0.1) at 0, 3, 6, 24, 48 and 72 hours post infection were measured by RV titration. RV concentration was compared with each time point post infection using a 1-way ANOVA ($n = 5$). (b,c) Time course of RV-induced IL-6 and IL-8. Concentration of (b) IL-6 and (c) IL-8 release from noninfected fibroblast (constitutive release) or UVi-RV-(UVi-) or RV-16-(RV-) infected fibroblasts (MOI = 0.1) at 0, 3, 6, 24, 48 and 72 hours post infection were measured by ELISA. RV-induced IL-6 and IL-8 at 48, and 72 hours post infection compared to control and UVi (2-way ANOVA, $n = 5$). All data are presented as mean ± SEM. Significance of comparisons is represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$.

infected with RV was analysed by ELISA after 48 hours and RV titration 24 hours post infection.

As before RV induced IL-6 and IL-8 (Figure 3, $P < 0.05$, $n = 9$-10). BAY significantly inhibited the constitutive release (Table 3) and RV-induced IL-6 at 10^{-6} M but failed to inhibit IL-8 at the concentrations used (Figures 3(a) and 3(b), $n = 10$, $P < 0.05$). DMF had no effect on RV-induced IL-6 and IL-8 (Figures 3(c) and 3(d), DMF: $n = 9$). Interestingly, DMF increased the constitutive release of IL-8 (Table 3, $n = 9$, $P < 0.05$). The highest concentration of vehicle used to dissolve BAY and DMF had no effect on the level of IL-6 and IL-8 induction.

BAY had no effect on RV replication ($n = 10$, data not shown). DMF, however, significantly increased RV replication at 10^{-8}–10^{-7} M (Figure 4, $n = 14$, $P < 0.05$). The highest concentration of vehicle used to dissolve BAY and DMF had no effect on RV replication.

3.4. Addition of a NF-κB Inhibitor to the Combination Therapy of a Corticosteroid and β_2 Agonist Inhibits RV-Induced IL-6 in Primary Airway Fibroblasts. Since BAY was able to inhibit RV-induced IL-6 release but not IL-8, BAY at 10^{-6}M was combined with the lowest concentration of salmeterol (Sal) (10^{-8} M) and fluticasone (Flut) (10^{-10} M) which caused an effect, to examine if the combination could inhibit RV-induced IL-6 and RV replication.

As before RV induced IL-6 (Figure 5, $n = 4$, $P < 0.05$). Salmeterol and fluticasone in combination (Sal + Flut) inhibited RV-induced IL-6 by 80%, and in the additional presence of the NF-κB inhibitor BAY (Sal + Flut + BAY), total inhibition occurred (100%) (Figure 5, $n = 4$, $P < 0.01$). However RV replication was not altered ($n = 4$, data not shown). The highest concentration of vehicle used to dissolve Sal + Flut and Sal + Flut + BAY had no effect on IL-8 induction or RV replication (data not shown).

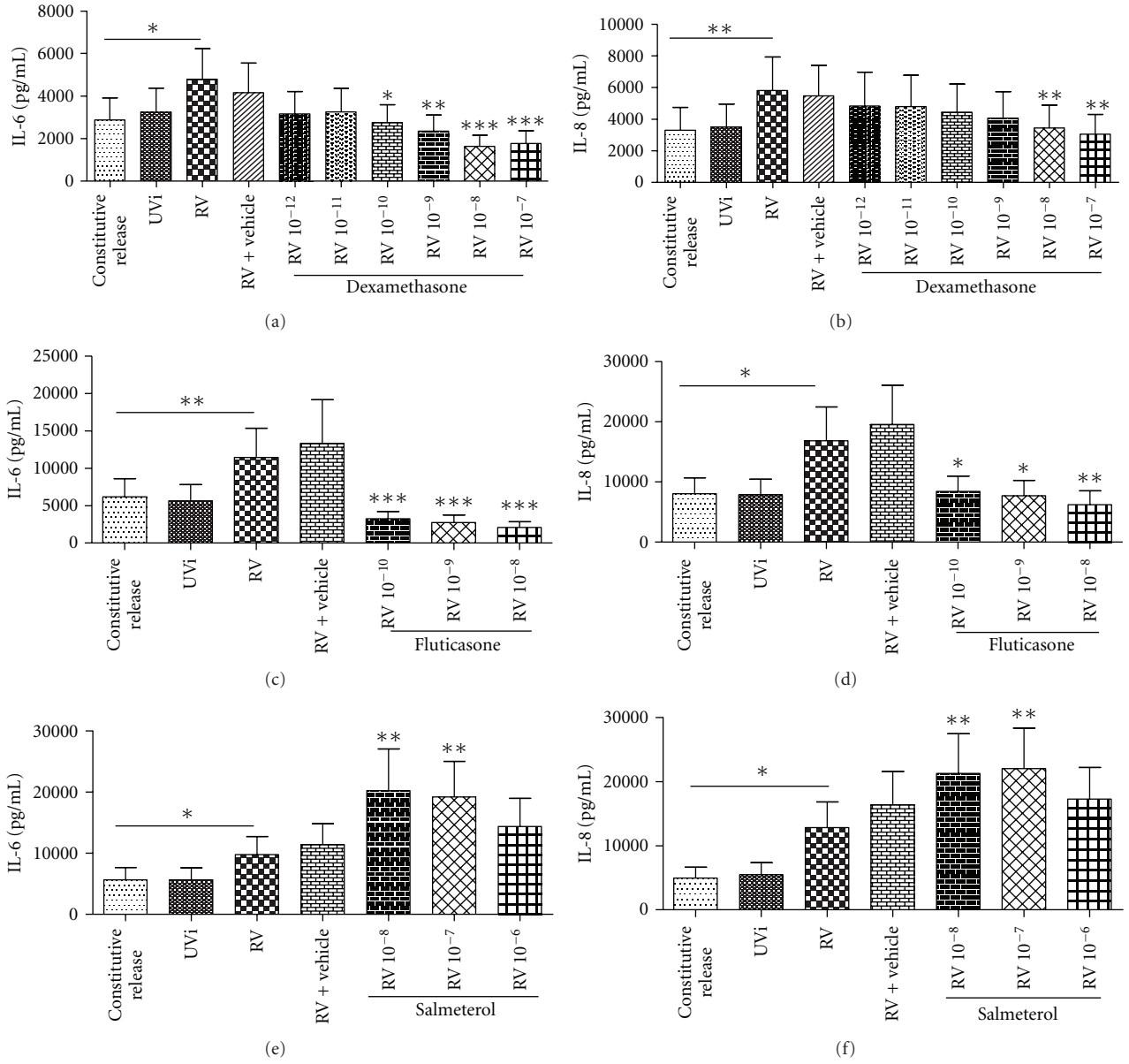

Figure 2: (a–f) Effect of dexamethasone (Dex), fluticasone (Flut) and salmeterol (Sal) on RV-induced IL-6 and IL-8. Concentration of IL-6 and IL-8 release from noninfected fibroblasts (constitutive release), UVi-RV-(UVi-) or RV-16-infected fibroblasts (RV) (MOI = 0.1), highest concentration of vehicle (Dex & Sal: 0.1% DMSO; Flut: 0.001% DMSO) and RV infected fibroblasts in the presence of Dex: 10^{-12}–10^{-7} M ($n = 7$), Flut: 10^{-10}–10^{-8} M ($n = 7$) and Sal: 10^{-8}–10^{-6} M ($n = 9$) were measured 48 hrs post infection by ELISA. All IL-6 and IL-8 concentrations were compared to their respective RV-induced values (in the absence of drug and vehicle), using a 1-way ANOVA. All data are presented as mean ± SEM. Significance is represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$.

4. Discussion

This study examined the effects of current asthma medications such as corticosteroids and β_2 agonists and potential novel treatments such as NF-κB inhibitors, as well as their combination in the treatment of RV-induced inflammation and RV replication in airway fibroblasts. The study confirmed that RV was able to infect and replicate in primary airway fibroblasts and that RV can induce proinflammatory cytokines IL-6 and IL-8 but not IL-28A and IL-29 from primary airway fibroblasts. Therefore this *in vitro* model simulates a possible underlying inflammatory scenario experienced during RV-induced asthma exacerbations *in vivo*. No induction was observed with UVi-RV, and thus UVi-RV-treated fibroblasts were not studied further.

Interferons are cytokines which are released by cells in response to pathogens to trigger protective defences of the immune system, and it has been shown that asthmatic patients may be more susceptible to RV infection due to their deficient interferon β and λ responses in bronchial epithelial cells [5, 17]. In our study we measured both IL-28A and

Table 2: Effects of dexamethasone (Dex), fluticasone (Flut), and salmeterol (Sal) on the constitutive release of IL-6 and IL-8.

	Constitutive release [pg/mL]			10^x [M]						
Drug	Cytokine	Constitu-tive release	Vehicle	−12	−11	−10	−9	−8	−7	−6
Dex	IL-6	2874 ± 1033	2974 ± 1078	3295 ± 1092	3734 ± 1369	3387 ± 913.8	2729 ± 834.1	1576 ± 567.4	1264 ± 380.6	
	IL-8	3305 ± 1429	3424 ± 1534	3277 ± 1521	3376 ± 1548	3499 ± 1391	3206 ± 1220	2590 ± 1240	2492 ± 1182	
Flut	IL-6	6195 ± 2429	6187 ± 2400			1712 ± 545.8***	1286 ± 375.0***	1123 ± 355.5***		
	IL-8	8035 ± 2609	8639 ± 2977			3334 ± 988.1*	2565 ± 695.6**	2681 ± 763.0*		
Sal	IL-6	5646 ± 2009	6620 ± 2438					9440 ± 4112*	7903 ± 2955	6277 ± 2335
	IL-8	4962 ± 1698	5974 ± 2023					8186 ± 3583**	6122 ± 2226*	5196 ± 1807

Values are means ± SEM.
* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

Table 3: Effects of BAY and DMF on the constitutive release of IL-6 and IL-8.

		Constitutive release [pg/mL]		10^x [M]		
Drug	Cytokine	Noninfected	Vehicle	−8	−7	−6
BAY	IL-6	5159 ± 1531	5005 ± 1631	5826 ± 1879	4790 ± 1530	2556 ± 803.9**
	IL-8	5598 ± 2109	4573 ± 1947	4442 ± 1761	3958 ± 1719	3423 ± 1334
DMF	IL-6	5743 ± 1001	5970 ± 1226	5907 ± 1086	5998 ± 1122	5937 ± 984.7
	IL-8	6151 ± 1055	7296 ± 1803	7180 ± 1664	7151 ± 1756	8329 ± 1780*

Values are means ± SEM.
*$P < 0.05$, and ***$P < 0.0001$.

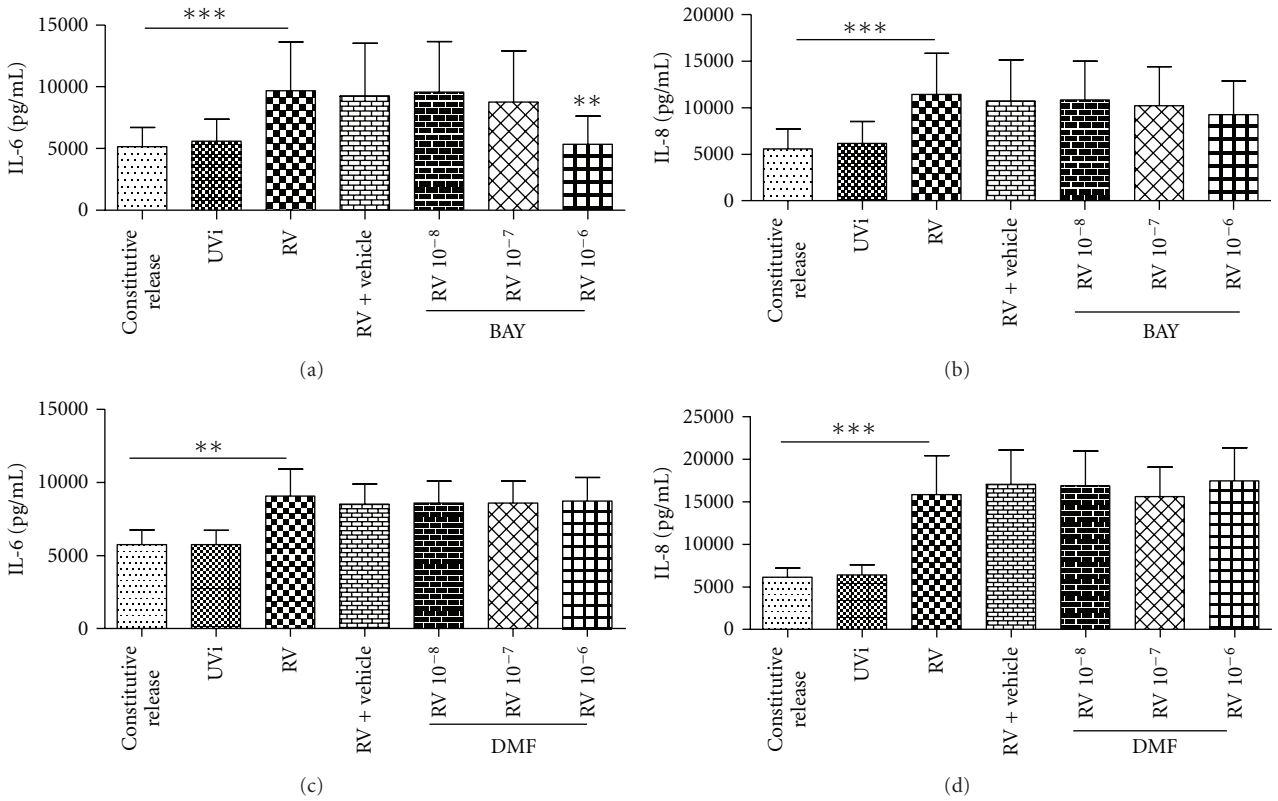

Figure 3: (a–d) Effect of BAY and DMF on RV-induced IL-6 and IL-8. Concentration of IL-6 and IL-8 release from noninfected fibroblast (constitutive release), UVi-RV-(UVi-) or RV-16-infected fibroblasts (RV) (MOI = 0.1), highest concentration of vehicle (0.1% DMSO) and RV infected fibroblasts in the presence of 10^{-8}–10^{-6} M BAY ($n = 10$) and DMF ($n = 9$) measured 48 hrs post infection by ELISA. All IL-6 and IL-8 concentrations were compared to their respective RV-induced values (in the absence of drug and vehicle), using a 1-way ANOVA. All data are presented as mean ± SEM. Significance is represented as **$P < 0.01$ and ***$P < 0.0001$.

IL-29 (2 members of the interferon-λ family) and found that RV infection of primary human fibroblasts does not induce interferon-λ above the level of constitutive release. This indicates that the production of interferons in response to RV is cell type specific, and our results are similar to other findings that showed RV does not induce interferon β in primary human fibroblasts [18], supporting their hypothesis that this susceptibility to RV infection may cause fibroblasts to act as reservoirs for RV replication and spread to the lower airways.

Toll-like (TLR) receptors are a class of receptors which recognise distinct molecular patterns that are shared by pathogens but not by the host. RV is a single-stranded RNA virus, which in theory could be detected by both TLR 7/8 (single-stranded RNA) and TLR 3 (double-stranded RNA) as replication occurs. In fibroblasts the mechanism by which RV induces cytokines is not known. In our experiments UVi-RV did not induce cytokines, suggesting that cytokine induction is replication dependent (i.e., the cell is detecting and responding to double-stranded RNA). Similarly, in bronchial epithelial cells, RV induces cytokines via the activation of TLR-3 and not TLR 7/8 [19]. However, this response is likely specific to RV as in our previous studies we have shown that fibroblasts respond to agonists of TLR-3 and TLR 7 and 8 [20]. TLR signalling pathways have not been extensively studied in lung fibroblasts; however, their activation leads to

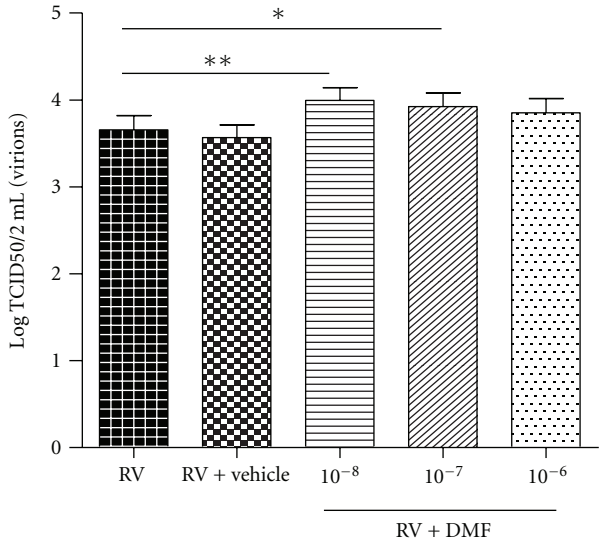

Figure 4: Effect of DMF on RV replication. Concentration of virus from RV-infected fibroblasts ± vehicle (0.1% DMSO); or 10^{-8}–10^{-6} M DMF ($n = 14$) was measured 24 hrs post infection by RV titration. All RV concentrations were compared to RV concentration in the absence of drug and vehicle by 1-way ANOVA. All data are presented as mean ±SEM. Significance is represented as $^{*}P < 0.05$ and $^{**}P < 0.01$.

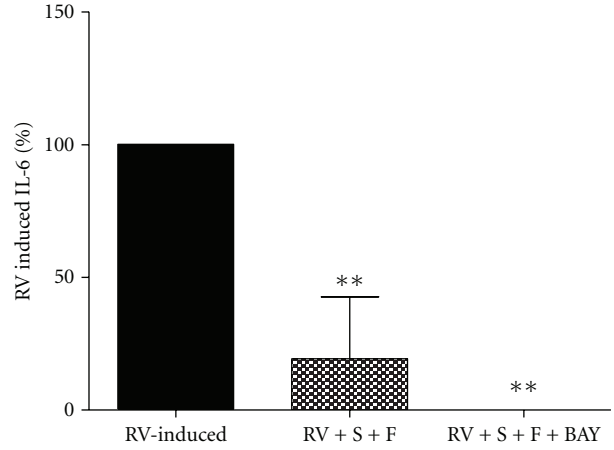

Figure 5: Salmeterol + fluticasone (Sal + Flut) and Sal + Flut + BAY inhibit RV-induced IL-6: the amount of IL-6 induced from fibroblasts infected with RV-16 (MOI = 0.1) was expressed as 100%. Inhibition caused by drug combinations, Sal + Flut (10^{-8} + 10^{-10} M) and Sal + Flut + BAY (10^{-8} + 10^{-10} + 10^{-6} M) (for all $n = 4$) was measured 48 hrs post infection by ELISA and expressed as a percentage of RV-induced IL-6. Percentage inhibition caused by drugs was compared using 1-way ANOVA with RV-induced IL-6. All data are presented as mean ± SEM. Significance is represented as $^{**}P < 0.01$.

downstream activation of transcription factors such as NF-κB, and this results in the upregulation and induction of various inflammatory cytokines such as IL-6 and IL-8 [21].

The study showed that, at a concentration of 10^{-10} M, fluticasone inhibited both RV-induced IL-6 and IL-8, while dexamethasone inhibited only IL-6. Fluticasone is a more potent corticosteroid in inhibiting inflammation [22], and our data reflect this fact. It is also interesting to note that, although IL-8 is released more abundantly than IL-6 [23], this selective inhibition suggests that IL-8 may at least in part be steroid insensitive, and other studies have also demonstrated that certain cytokines are steroid insensitive [24, 25]. Nevertheless, to our knowledge this study is the first to report of RV-induced IL-6 and IL-8 inhibition by corticosteroids in primary airway fibroblasts and confirms previous studies demonstrating that corticosteroids inhibits RV-induced cytokines [7, 26].

The major function of both long- and short-acting β_2 agonists in the treatment of asthma is to maintain or to induce airway relaxation. β_2 adrenoceptors are present on lung fibroblasts as well as airway smooth muscle (ASM); therefore, β_2 agonists may affect fibroblast activities [27]. Previous studies have produced conflicting results in regards to the effects of β_2 agonists on cytokine induction in various airway cells. β_2 agonists increased the secretion of TNF-α- and TGF-β-induced IL-6 in ASM, had no effect on RV-induced IL-8 in bronchial epithelial cells, and inhibited IL-4 in human peripheral blood mononuclear cells and inhibited cytokine-induced adhesion molecule expressions, such as ICAM-1 in human lung fibroblasts [25–31]. This suggests that β_2 agonists *in vitro* can have positive, neutral, or even negative effect on cytokine induction in various airway cells and that the effects of β_2 agonists may be stimulus and cell type dependent. The current study showed for the first time that β_2 agonists further increased RV-induced IL-6 and IL-8. The mechanism by which β_2 agonists increase inflammation may be explained by their mechanism of action at the cellular level. β_2 agonists stimulate the β_2 adrenoceptor and activate adenyl cyclase which gives rise to an increase in intracellular cAMP levels which binds to the cAMP responsive element binding protein (CREB) in the promoter region of genes and can result in upregulation of various genes [29]. Since RV infection alone induces cytokines, the use of β_2 agonists may result in a second signal to further induce proinflammatory cytokines.

Despite their inflammatory modulatory capacity, neither corticosteroids nor β_2 agonists affected RV replication and this suggests that RV replication may not be dependent on RV-induced inflammation. However *in vivo* studies have shown that intranasal use of corticosteroids increased and prolonged RV number and shedding which may be due to the presence of the immune system [32].

There is good evidence suggesting that RV-induced inflammation is due to NF-κB activation [6, 33]. For this reason, this study is the first to have examined the effects of inhibiting NF-κB on RV-induced IL-6 and IL-8 and RV replication.

BAY inhibits NF-κB by inhibiting IκB-α phosphorylation [34]. The study showed that BAY inhibited RV-induced IL-6 but not IL-8 and this was unexpected as previous studies found that transcription of IL-6 and IL-8 is regulated by the same transcription factors: NF-κB, AP-1, CREB protein, and CCAAT/enhancer binding protein (C/EBP) [25, 35].

Although transcription of IL-8 is mediated by the same transcription factors as IL-6, although IL-8 is partially regulated by NF-κB, it may be more dominantly or synergistically regulated by other transcription factors such as AP-1 or C/EBP and hence explains the result [16, 36]. This study showed that the inhibitory effects of BAY on RV-induced IL-6 and IL-8 were not as effective as the corticosteroids.

DMF inhibits the translocation and partially inhibits the transactivation of NF-κB but does not inhibit NF-κB completely [37]. In our study, DMF had no effect on RV-induced IL-6 and IL-8 but increased RV replication. The increase in replication may be an example of how some viruses can exploit NF-κB for their own replication and survival [8]. Most viruses that induce NF-κB activity often harbour NF-κB binding elements in their viral promoters and therefore would have a replicative advantage if there is active NF-κB in the cytoplasm [8, 38]. One example of this is the low-level NF-κB activation by HIV-1 which allows HIV-1 to maintain a chronic infection in myeloid cells [38]. By using the basic local alignment search tool (BLAST) we found that there are a few NF-κB binding motifs on the RV genome [39]. Although we cannot confirm whether these binding motifs can actively bind NF-κB and produce a functional outcome, if they are true it may be possible that RV-16 may be utilising a similar process to HIV-1. Alternatively, DMF may have other properties which may be delaying cellular apoptosis and therefore allowing increased replication to occur [40], both, which require further investigation.

These results suggest that NF-κB activation alone may not be the factor that results in RV-induced inflammation but perhaps a combined activation of NF-κB, AP-1 and C/EBP, and alternative inhibitors of these transcription factors should be examined.

This study also examined whether the commonly used corticosteroid and β_2 agonist combination therapy in the treatment of asthma could affect RV-induced IL-6 and RV replication. Our study showed that fluticasone inhibited IL-6 production (100%) in response to RV infection, whilst salmeterol increased RV-induced IL-6 production. However when used together suppression of RV-induced IL-6 occurred (80%), reflecting similar results to Edwards et al., [2] showing the same combination inhibiting RV-induced IL-8 from bronchial epithelial cells. The addition of the NF-κB inhibitor BAY to this combination suppressed RV-induced IL-6 further (100%). Despite neither combination altering RV replication, the results were as expected, as earlier we established that both BAY and fluticasone were potent inhibitors of RV-induced IL-6, and this combined result shows the additive inhibitory effect of these drugs. These findings suggest that during RV-induced asthma exacerbations, the use of combination therapy may not be as useful as corticosteroids alone, but may still be beneficial. Furthermore, it is possible that incorporating a NF-κB inhibitor into this combination may make the combined therapy more effective for the treatment of both asthma and RV-induced asthma exacerbations.

This study albeit *in vitro* suggests that in the event of RV-induced asthma exacerbation, asthma steroidal medication alone is more beneficial in inhibiting RV-induced inflammation than β_2 agonists. In reality this is unrealistic as β_2 agonists are required to provide bronchorelaxation during RV-induced asthma exacerbations. Therefore perhaps alternative bronchodilators such as anticholinergics could be used during a viral exacerbation. Furthermore, NF-κB inhibitors are not as effective as corticosteroids and may be detrimental if used incorrectly. In conclusion, NF-κB inhibitors remain a potential therapeutic treatment for RV-induced asthma exacerbation; however, future research into drug combinations such as corticosteroids and NF-κB inhibitors, multiple NF-κB inhibitors or inhibitors of other transcription factors such as AP-1 and C/EBP may pave the way for more therapeutic options.

Conflict of Interests

The authors have no conflicting financial interests.

Acknowledgments

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