Allergen-induced growth of airway smooth muscle: structure-function relations and modulation by corticosteroids and the epidermal growth factor receptor

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Dedication

I have been very fortunate to have understanding parents who have always encouraged me to pursue my own dreams, a mentor who believed in my potential and helped to groom me into a more confident critical thinker, and an entire family and supportive group of friends that have been encouraging throughout the years and without whom, life would just not be the same.

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Contribution of Authors

This document has been prepared as a manuscript-based thesis, consisting of text from manuscripts that have either been published, submitted for review, or are to be submitted for review for subsequent publication.

Chapter 2: The site of airway narrowing during the early allergic response and the influence of exogenous surfactant

Dr Kimitake Tsuchiya (post-doctoral fellow, PDF) contributed to setting up the early allergic response (EAR) model in the Brown Norway rat and helped to perform a number of the physiological assessments of the EAR, staining of tissue sections with hematoxylin and eosin and morphometric analysis.
Dr Paul-André Risse (PDF) contributed by suggesting and performing the experiments to quantify the effects of surfactant on the calcium release from mast cells.
Dr Sharon Bullimore (PDF) contributed the figure (Figure 8) on the effects of airway size on surface tension for this project.
Dr Andrea Benedetti performed the statistical analysis for the EAR project.
Dr James G. Martin contributed to the design of the study, reviewed the data analysis, contributed to the interpretation of the findings and the writing of the manuscript.

Chapter 3: Sites of allergic airway smooth muscle remodeling and hyperresponsiveness are not associated in the rat

Dr Taisuke Jo (PDF) helped in performing animal experiments. He also provided statistical advice and performed statistical analyses on data in a number of the figures.
Dr Meiyo Tamaoka (PDF) assisted with the experimental preparation at the beginning of the study.
Dr Heberto Ghezzo was the primary statistician for this chapter.

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Chapter 4: The potential modulation of the airway smooth muscle phenotype and the effects of the tyrosine kinase inhibitor AG1478 in a repeated allergen challenge model of asthma

Dr Mauro Novali (PDF) helped in sectioning and staining slides for subsequent laser capture microdissection.
Dr Kimitake Tsuchiya (PDF) helped in performing laser capture microdissection and RT-PCR.
Dr Paul-André Risse (PDF) provided assistance in performing RT-PCR, designed primers and helped in analysis of some AHR data.
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Miss Cinzia Marchica (MSc) helped with the initial airway smooth muscle analysis.
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Table of Contents

Abstract

Allergen challenges cause airway structural changes, termed *remodeling*, including an increase in smooth muscle (SM) mass and goblet cell metaplasia. These changes are substantially mediated via the epidermal growth factor receptor (EGFR) and its axis of ligands. The causal relationship between remodeling and another key asthmatic feature, airway hyperresponsiveness (AHR) has not been clearly established. The first objective was to investigate the site of airway narrowing in response to a single allergen challenge. We used two approaches, a morphometric assessment and an analysis of respiratory mechanics, using the constant phase model to partition the site of responses. We snap froze lungs during the early allergic airway response (EAR) and performed a morphometric analysis of airway luminal areas. Interestingly, the EAR response demonstrated an initial, transient response in the large airways yet, a more sustained response in the peripheral lung. We reasoned that in peripheral airways, the perturbation of the surface tension of the airway lining fluid by protein leak from the vasculature may have contributed to the response. The role of exogenous, natural surfactant in the EAR has not been explored extensively. The surfactant used largely contained surfactant protein (SP)-B but lacked SP-A and SP-D, both of which confer immunomodulatory properties on surfactant. We confirmed that effects of the surfactant may not have been purely via physical properties but also by immunomodulation. This notion was confirmed as surfactant inhibited the mast cell mediators, cysteinyl-leukotrienes and amphiregulin, *in vivo.*

We next investigated the relationship between allergen-induced AHR and airway remodeling. We induced airway remodeling using the Brown Norway rat undergoing three multiple challenges with the sensitizing antigen ovalbumin. We used the constant phase model to evaluate mechanical responses to inhaled aerosols of methacholine. AHR in the peripheral lung compartment and airway remodeling across all airway sizes were observed 48 h after the last of three allergen challenges. The corticosteroid budesonide (0.1mg/kg), used to further

probe the relationship between airway remodeling and AHR, was sufficient to inhibit remodeling but not AHR. At one week, AHR did not persist despite the remodeling.

Finally we examined the contractile SM properties 48 h after the last allergen challenge and studied whether inhibition of the EGFR modulated the SM phenotype and AHR. We used laser capture microdissection to harvest SM and epithelial cells for the assessment of mRNA expression. As earlier observed, AHR was induced, but only in the periphery despite increases in SM-myosin heavy chain (sm-MHC) gene expression and growth of SM in the larger airways. EGFR inhibition decreased all these outcomes in addition to SM growth across all airways and inflammation.

In conclusion, the site of allergic airway narrowing is initially located in the central airways and over time develops in the lung periphery. Modulation of surface tension influences the magnitude of airway narrowing during the EAR. After multiple allergen challenges, AHR is placed in the peripheral airways exclusively despite airway remodeling in the central airways and increased sm-MHC gene expression in the muscle in these airways, all of which were reduced by EGFR inhibition. Our studies show that the relationship between airway remodeling and AHR is not a simple one and the two phenomena seem to be dissociated in the rat model of allergic asthma.

Résumé

L'exposition chronique aux allergènes provoque des changements structurels des voies respiratoires qualifiés de remodelage. Ces changements sont substantiellement véhiculés par le récepteur du facteur de croissance épidermique (EGFR) et de ses ligands. La relation entre le remodelage et l'hyperréactivité bronchique (HRB), un autre élément clé de l'asthme, n'a pas été clairement établie.

Le premier objectif de ce travail était de définir le site de l'obstruction des voies aériennes, induite par une exposition allergénique. Nous avons utilisés deux approches, l'analyse morphométrique des voies aériennes et la mesure des mécaniques respiratoires en utilisant le modèle de phase constante. L'analyse morphométrique indique que les voies aériennes périphériques sont responsables de l'HRB induite par l'allergène. La réponse allergique immédiate (RAI) était associée à une bronchoconstriction transitoire dans les larges voies respiratoires, suivi d'une réponse dans le compartiment périphérique du poumon. Nous avons émis l'hypothèse que cette réponse pourrait résulter d'une perturbation des tensions de surface des voies aériennes périphériques due à une extravasation de protéines plasmatiques. La stabilité des voies aériennes périphériques peut être influencée par les protéines du surfactant. L'apport exogène de protéines de surfactant naturel a été exploré. Le surfactant utilisé était majoritairement composé de protéines B du surfactant, SP-B, mais ne contenait ni SP-A ni SP-D aux propriétés immunomodulatrices. Nous avons démontré que le surfactant avait des effets physiques mais aussi pouvait être immunomodulateur. La réduction des concentrations des médiateurs mastocytaires, les leukotriènes et l'amphiréguline, dans les lavages broncho-alvéolaires confirme cette notion.

Dans le but de mieux comprendre la relation entre remodelage et HRB, nous avons induit le remodelage bronchique chez des rats BN provoqués chroniquement à l'ovalbumine. Les mesures de mécanique respiratoire ont été réalisées en appliquant le modèle de phase constante afin de distinguer les sites pulmonaires impliqués dans la bronchoconstriction induite par des doses croissantes de métacholine. Deux jours après la dernière des trois provocations L'HRB était présente dans le compartiment périphérique du poumon alors que le

remodelage était présent dans toutes les voies aériennes. Le budésonide, un anti-inflammatoire corticostéroïdien utilisé pour mettre en évidence la relation entre l'HRB et le remodelage, a inhibé le remodelage sans affecter l'HRB. A l'inverse du remodelage, L'HRB n'a pas persistée après une semaine.

Finalement le phénotype contractile du muscle lisse (ML) et le rôle du récepteur EGFR ont été étudiés dans le modèle de provocations allergénique répétées. Nous avons utilisé la capture par microdissection laser pour évaluer l'expression génique dans le ML et l'épithélium. Comme précédemment observé L'HRB a été induite uniquement dans les voies aériennes périphériques bien que l'expression du gène de la chaîne lourde de myosine et l'hyperplasie du ML aient été observées dans les larges voies aériennes. L'inhibition du récepteur EGFR a réduit l'expression de la chaine lourde de myosine, l'HRB et le remodelage du ML ainsi que l'inflammation.

En conclusion le site de l'obstruction bronchique est initialement localisé dans les voies aériennes centrales avant de se développer en périphérie. La modulation de la tension de surface influence l'amplitude de l'obstruction bronchique durant la RAI. Les provocations allergéniques répétées entraine une HRB exclusivement périphérique malgré l'apparition d'un remodelage du ML et d'une augmentation d'expression de la chaine lourde de myosine dans les voies aériennes centrales. Toutes ces dernières manifestations ont été réduites par l'inhibition du récepteur EGFR. Nos travaux démontrent une relation complexe entre HRB et remodelage, deux phénomènes dissociés dans le modèle d'asthme allergique chez le rat.

Chapter 1

Introduction and Literature Review

1.1 **Introduction**

The basic function of the lung is to cater to the body's needs for oxygen uptake and carbon dioxide elimination. The lung consists of a bifurcating tree of airways that conducts gas in and out of the parenchyma comprising the gas exchanging alveolar compartment. As the airways branch progressively along the airway tree, their numbers increase geometrically. As a result, the total cross-sectional area increases almost exponentially with increasing distance from the primary bronchi with a concomitant decrease in axial airflow velocity during ventilation. The airways at the furthest distance from the primary bronchi gradually evolve in their functions, from gas conducting to gas diffusing in the respiratory bronchioles.

The human lungs show asymmetry; the right lung comprises three lobes whereas the left lung is constituted of two lobes. Similarly, a rat lung has four lobes on the right but only one lobe on the left. The human trachea branches into two main bronchi which individually enter either the right or left lung. Both the trachea and main bronchi contain cartilaginous rings. As one moves more distally, at the level of the bronchioles, cartilage is no longer present and alveoli begin to appear in the walls of the respiratory bronchioles, and they continue to increase in number in the alveolar ducts which are even more distal and the airways terminate in several grapelike clusters made entirely of alveoli.

The airway wall can be divided into three compartments: (i) the inner wall, epithelium, basement membrane, and lamina propria, (ii) the outer wall, consisting of cartilage and connective tissue between the muscle layer and the surrounding parenchyma, and finally, (iii) the smooth muscle (SM) layer (143). Changes in the volume fractions of these compartments have important implications for airway function. In this context, the proposed nomenclature by Bai and colleagues places the SM layer within the inner wall (7). The SM surrounding airways may alter the radii of airways by contracting or relaxing. SM contraction leads to airway narrowing and increases the impedance to airflow. Excessive SM contraction causing a sufficiently diffuse airway narrowing which lead to symptoms appears to be the root cause of asthma (2). Thickening of the

airway wall components can be observed in all-sized airways in asthma (26; 42; 81; 103; 157; 184).

Asthma is a condition of the lung that has been agreed upon by the American Thoracic Society to be constituted of:

(i) reversible airway obstruction,

(ii) inflammation, which is often associated with high levels of eosinophils,

(iii) airway hyperreactivity, or hyperresponsiveness (AHR), which is reflective of enhanced airway narrowing and an enhanced response to a given stimulus, and more recently,

(iv) airway remodeling.

The majority of asthmatic sufferers are mildly affected but asthma can be severe and occasionally fatal. In allergic asthma, the response to allergen triggers the following cascade:

(i) initial sensitization,

(ii) the early allergic response,

(iii) the late allergic response

and upon repeated challenges;

inflammation, AHR, and airway remodeling.

This thesis addresses questions related to the mechanisms of airway narrowing in a rat model of allergic asthma, the development of airway remodeling and the relationship between airway remodeling and AHR.

1.2 **Sensitization**

Generally, the routes of exposure to allergen that may lead to sensitization are inhalation, ingestion or via the skin. In human allergic asthma, the process of sensitization leads to high levels of circulating allergen-specific immunoglublin E (IgE) that reflect a deviated Th2 immune response. There is a range of common aeroallergens among which are house dust mite (HDM), ragweed, birch pollen, cat and dog (74). This process of Th2 sensitization to inhaled allergens often

occurs at a very young age and is influenced by genetic and environmental factors such as childhood viral infections and environmental exposure to microbial compounds (77; 173; 183; 194).

Sensitization is likely to occur when dendritic cells (DCs) reach full maturity upon their encounter with allergens. Exposure to lipopolysaccharide (LPS) and viral infection recruits immature myeloid DCs to the airways where they may sample the lumen for potentially antigenic proteins. They undergo their full maturation during migration to the draining lymph nodes (110). DCs serve as professional antigen presenting cells, by efficiently capturing and processing antigens, expressing lymphocyte co-stimulatory molecules, and migrating to lymph nodes and providing the signal for naive T cells to proliferate and acquire Th2 effector function (75). T cells provide help to antigen-specific B cells, which subsequently develop into plasma cells and produce and secrete antibodies (IgE) specific to the allergen. IgE binds to receptors on the mast cells and basophils through high affinity IgE receptors. When a second encounter with antigen occurs, the binding to IgE with cross-linking of adjacent IgE molecules leads to cellular activation and the cascade of reactions that typifies an allergic response.

There are several dendritic cells with distinct lineages. Myeloid dendritic cells appear to be most relevant to allergic responses whereas plasmacytoid dendritic cells (pDCs) are tolerogenic. It is possible that a suppression of the tolerogenic potential of plasmacytoid DCs may contribute to the process of sensitization. Allergic children have been found to have significantly fewer circulating pDCs compared with nonallergic children (58). Secondly, lower respiratory tract viral infection modulates sensitization to allergens but also strongly interacts with pDC function (76). As part of their normal function as natural interferon-α producing cells, pDCs undergo functional maturation during viral infection and become immunogenic rather than tolerogenic cells (5).

1.3 **The early allergic response (EAR)**

The early response is the consequence of the immunological reaction which occurs immediately upon re-exposure of sensitized subjects to allergen and is also

observed in the rat (40) as well as a number of other species (12; 156). It involves immunoglobulin E (Ig-E)-mediated mast cell activation and the subsequent release of mediators of bronchoconstriction from mast cells (93; 135). While being bound to its receptors on mast cells through its Fc fragment, IgE binds to the allergens through its Fab portion and when two adjacent mast-cell-linked IgE antibodies are in place, the cross-linking of the two IgEs triggers a reaction. When sufficient IgE molecules are cross-linked, the mast cell releases mediators of bronchoconstriction including the pre-stored biogenic amines histamine/serotonin and *de novo* synthesis of cysteinyl-leukotrienes (leukotriene C_4 that stimulates mucus secretion, bronchial SM contraction and microvascular leak) and the lipid mediator prostaglandin D_2. These mediators contribute to the wheezing experienced by those sensitized subjects that are exposed to pertinent allergens. Mast cells synthesize a range of cytokines, particularly those associated with Th2 type response such as IL-4, IL-5, IL-13 and TNF-α and amphiregulin. In addition, individuals may sneeze, cough, have itchy eyes and rhinorrhea that reflect rhinoconjunctivitis and become short of breath as a result of bronchospasm.

Mast cells are present in large numbers in most body tissues (such as the skin, nose, lungs and the gastrointestinal tract). Mast cells do not migrate into the lumen and are sparsely present in the bronchoalveolar lavage (BAL) fluid in animals. More than 90% of lung mast cells express a "mucosal" phenotype instead of a connective tissue phenotype as they express tryptase only, and not chymase (164). Mucosal mast cells have a substantially greater potential to synthesize leukotrienes than connective tissue mast cells and presumably have more importance in mediating allergic airway responses.

Leukotrienes are potent mediators of inflammation, derived from arachidonic acid through the 5-lipoxygenase pathway (5-LO) pathway. Release of 5-LO products leukotriene B_4 (LTB_4), the cysteinyl leukotrienes (CysLTs) C_4, D_4, E_4, and cycloxygenase products prostaglandin D_2 (PGD_2) and thromboxane A_2 from inflamed airways leads to AHR and airflow obstruction from smooth muscle contraction, microvascular leak of fluid and mucus glycoproteins.

Evidence from animal models and patients with asthma suggests that these eicosanoids are key molecules that promote airway inflammation as chemoattractants for eosinophils, T cells, and other inflammatory cells. They also cause plasma extravasation and edema, and modulate structural cells such as ASM from which they induce the release of extracellular matrix components (67; 68; 177). They have been extensively implicated in airway remodeling.

The cys LTs are potent bronchoconstrictors but also cause an increase in vascular permeability with the escape of plasma into the airway lumen (47; 137). Microvascular leak has important consequences for the mechanics of airway narrowing because the presence of protein within the airway lumen disrupts surfactant function and in addition, surfactant phospholipid changes occur during allergen-induced airway reactions which may amplify this effect (72; 120). As discussed in greater detail below, the stability of small airways, in particular in small experimental animals, may require the presence of surfactant to reduce interfacial tension. As a corollary the increase in surface tension resulting from loss of surfactant may serve as an additional mechanism for airway narrowing following allergen challenge.

1.4 **The late allergic response (LAR)**

The LAR begins shortly after the early allergic response, and it has a longer time course, lasting from 3-10 hours after exposure to allergen in sensitized subjects. During the LAR, congestion and other symptoms can be more severe than those experienced by subjects during the EAR. While the EAR is more closely associated with mast cells, the LAR involves inflammatory cell recruitment, typically eosinophils (124) which are the immune effector cells usually associated with allergic reactions, and the LAR can last for 24 hours or so before subsiding. T cells have also been implicated in the LAR. In 1995, it was reported that there could be an adoptive transfer of late allergic airway responses with antigen-primed $CD4^+$ but not $CD8^+$ T cells using a Brown Norway rat model (187). Antigen-primed $CD8^+$ T cells were later demonstrated to have a potent suppressive effect on LAR (167). A follow-up study indicated that these cells

were likely $CD8^+$ γδ T cells whose secretion of IFN-γ potently inhibits allergen-induced airway eosinophilia and late allergic responses (79). Antigen-primed $CD8^+$ αβ T cells appear to have a proinflammatory role in the LAR and airway eosinophilia in the rat (80).

Interesting, the surfactant dysfunction resulting from the LAR is linked with a reduced level of the hydrophobic surfactant protein SP-B and thus, increased surface tension in mice (57). As mentioned in the context of the EAR, a loss of surfactant in the airways during the LAR could also act to narrow the airways, and most particularly the small airways. In this thesis we have explored the potential role of surface tension in allergen-induced airway narrowing through the administration of surfactant to allergen-challenged animals.

1.5 **Surfactant**

Pulmonary surfactant is a mixture of phospholipids, neutral lipids, and specific proteins including surfactant proteins A-D. Surfactant proteins A and D are known as collectins. They are known to have immunomodulatory effects while surfactant proteins B and C are commonly associated with maintaining the homeostasis of the lung. Phosphatidylcholine (PC), mainly in the disaturated dipalmitoylphosphatidylcholine (DPPC) form, is the most abundant phospholipid in surfactant. Other phospholipids include phosphatidylglycerol (PG), phosphatidylethanolamine (PEA), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), and lysophosphatidylcholine (LPC). Surfactant reduces surface tension, stabilizes alveolar walls and prevents collapse at low lung volumes (83). Although the major biological function of surfactant is to maintain the mechanical properties of the alveolar spaces and faciliate ventilation it may also serve to stabilize small airways. Surface tension within the small airways should favour airway narrowing (192) and the effect would be expected to be markedly enhanced by the disruption of surfactant properties by the microvascular leak of protein that has been shown to occur during the EAR (137).

Exogenous surfactant administration may be beneficial in restoring disrupted surfactant activity (95). In 2003, Babu et al., provided evidence in a

clinical study that pulmonary surfactant dysfunction may also contribute to the EAR and may be ameliorated by exogenous, synthetic surfactant that contains no surfactant proteins (6). These data support earlier findings by Kurashima et al. in humans (102) and in guinea pigs (101). On the other hand, a natural porcine surfactant reportedly augmented airway inflammation 24 hours (h) after allergen challenge in humans (45). The facilitation of allergen presentation to lung epithelial cells by exogenous surfactant may account for the unexpected results (53). Oxygen free radicals and plasma proteins may contribute to inhibition of the surfactant effects in acute asthma (109).

Forty-eight hours after antigen challenge, there is reportedly a dysfunction in pulmonary surfactant (82). In asthmatic humans, phospholipid alteration in surfactant, particularly in phosphatidylglycerol, occurs after antigen challenge (72). Surfactant may be exerting its effects via its primary phospholipid constitutent, DPPC, adsorbing to the epithelium (chemisorption, or chemical adsorption) and thereby, suppressing, or masking, the sensitivity of bronchial irritant receptors and reducing afferent neural feedback to the brainstem (69). Interestingly, surfactant has also been proposed as a SM relaxant *in vitro* in the presence of an intact epithelium and its mediation of SM relaxation is by cyclooxygenase-dependent pathways (96). In summary, exogenous surfactant administration could serve as a useful adjunct in controlling allergen-induced symptoms in patients with allergic asthma.

1.6 **Airway inflammation**

Asthma is an inflammatory condition characterized by increased numbers of activated T lymphocytes, macrophages, eosinophils, basophils and mast cells in the airway tissues. Interestingly, airway inflammation, characterized by lymphocytic infiltration, was reported to be comparatively uniform in asthma compared to the variation seen in airway structure (26). The components of the peripheral inflammation in fatal asthma have been described by many investigators (41; 44; 48; 123; 157; 168). These studies highlight the substantial inflammatory infiltrate in peripheral airways in fatal asthma.

Regional variations in inflammatory cell distribution within the airway wall in patients with asthma were reported by Haley et al. (59). They reported that in airways larger than 3.0 mm basement membrane perimeter, patients had more eosinophils in the inner airway region (between basement membrane and SM) when compared to the same region in the relatively smaller airways (59). In smaller airways there was also an increased eosinophil number in the outer region (between SM and alveolar attachments) compared to the inner airway wall region (59). This group speculated that inflammatory cell density in peripheral airways in severe asthma may relate to the peripheral airway obstruction in asthma (59). Carroll et al., on the other hand, reported that increased numbers of lymphocytes are uniformly distributed in the large and small airways in cases of asthma, independently of asthma severity, and that eosinophil recruitment may be related to asthma severity (25). Hence, with these latter findings, biopsy specimens of proximal airways are likely to be representative of the cellular infiltrate in large and small airways from mild to severe asthma (25).

In the case that inflammatory cell infiltration is not uniform throughout the airway tree, there are a number of possibilities as to why this may be. Cell recruitment, retention and microenvironmental viability could be responsible for the differences in inflammatory cell infiltration. Large and small airways differ in blood supply; inner regions of the large airways are supplied by bronchial circulation, whereas the outer wall and small airways are supplied by the pulmonary circulation (38). Vessels may have different distinctive expression of endothelial adhesion molecules that, in turn, could promote different degrees of inflammatory cell recruitment. There could be spatial differences amongst airways of different sizes to produce cytokines that influence inflammatory cell infiltration and thus, the milieu. Also, there may be regional differences in innervation (8). This could result in regional differences in responses to those stimuli having effects through inflammatory mediators, including cytokines and chemokines, as well as through activation of C-fiber afferents, leading to release of neuropeptides such as the neurokinins.

1.7 **Airway remodeling**

The collective term 'airway remodeling' refers to long-term structural airway changes resulting from multiple allergen exposures. The structural cells that are most affected include the epithelial cells, the cells closest to the airway lumen, airway smooth muscle cells found in bundles further into the adventitia, subepithelial fibroblasts and myofibroblasts, cells pertaining to the vessels such as endothelial and vascular smooth muscle cells, and nerve cells. The structural changes caused in the airways by multiple allergen exposures consist of, but are not limited to, epithelial proliferation, epithelial shedding and fragility, goblet cell differentiation, subepithelial fibrosis, airway smooth muscle (ASM) growth, angiogenesis, matrix protein deposition, and mucus gland hyperplasia and hypertrophy.

Airway remodeling and airway hyperresponsiveness, two of the primary features of asthma have been of principal interest to us and understanding their relationships is the underlying theme for this thesis.

1.7.1 **Airway remodeling-epithelium**

The epithelium comprises the initial barrier to all foreign inhaled particles, gaseous irritants or infectious agents. The epithelium is naturally equipped with a variety of mechanisms to defend against a potentially harmful milieu. It can be activated via a range of pathogen recognition receptors including the Toll-like receptors (TLRs) that may induce the secretion of various pro-inflammatory molecules. Descriptive studies using human airway tissues, *in vivo* and *in vitro* models have been used to study epithelial remodeling. Epithelial cell hyperplasia occurs after allergen challenge in animals (151). Epithelial shedding also occurs in allergen-driven animal models but the mechanisms are not entirely clear. The epithelium also has the capacity to differentiate into mucus-producing and mucus-secreting goblet cells. The asthmatic epithelium is fragile and many viable bronchial epithelial cells may be shed into the lumen and may also be detected in asthmatic expectorated sputum.

Murine models are not suited to model mucus gland hyperplasia as they have no glands except in the upper trachea. There are several important mediators of epithelial proliferation and goblet cell metaplasia including interleukin (IL)-13, IL-9, neutrophil elastase, cysteinyl-leukotrienes and, most recently, γ-aminobutyric acid, an amino acid transmitter which mediates rapid inhibition in the central nervous system (3; 28; 180; 182; 191). Many of these mediators appear to be upstream of the epidermal growth factor receptor (EGFR), which is activated by potentially different mechanisms depending on the stimulus.

1.7.2 Epidermal growth factor receptor (EGFR)

The EGFR (ErbB-1) has been a receptor of interest for our laboratory over recent years. It is a tyrosine kinase receptor belonging to a family of four tyrosine kinase (ErbB-1, ErbB-2, ErbB-3, ErbB-4) receptors. A lack of these receptors in mice results in lethality (125). The EGFR was discovered by Stanley Cohen of Vanderbilt University and Rita Levi-Montalcini for which both of them received the 1986 Nobel prize in Physiology or Medicine. The EGFR has been widely described to be important in the malignant behaviour of some tumors (50). Up-regulation of the EGFR has been described in asthmatic airway tissues (4; 150). One report from our laboratory showed an upregulation of heparin-binding EGF (HB-EGF) in ASM in severe asthma (63). EGFR is commonly expressed on the epithelium, airway smooth muscle, basement membrane and glands (4). Additionally, in 2004 it was reported that EGFR was not expressed on T cells and peripheral blood mononuclear cells (PBMCs) (85). The ligands commonly associated with the EGFR are amphiregulin, betacellulin, epidermal growth factor (EGF), HB-EGF, epigen, epiregulin, transforming growth factor-α (TGF-α). However, HB-EGF, betacellulin, and epiregulin have also been reported to be ligands for ErbB-4. Upon ligand binding, once EGFR is activated, upon phosphorylation, it dimerizes and adapter proteins are recruited for downstream MAPK signaling.

EGFR has been implicated in wound healing repair involving the epithelium (150) in airway smooth muscle (ASM) growth, as is expected given

that EGF is a potent mitogen for ASM, and mediating mucus production as well as mediating airway hyperresponsiveness (AHR) and inflammation via leukotrienes (178). The role of EGFR in potentially modulating ASM phenotype has not yet been studied and has been of interest to us. In our studies we have studied the role of EGFR by using a tyrphostin, AG1478, which inhibits the phosphorylation of this receptor. We have also studied how it differentially may have effects on ASM growth and AHR in the central versus peripheral regions of the lung.

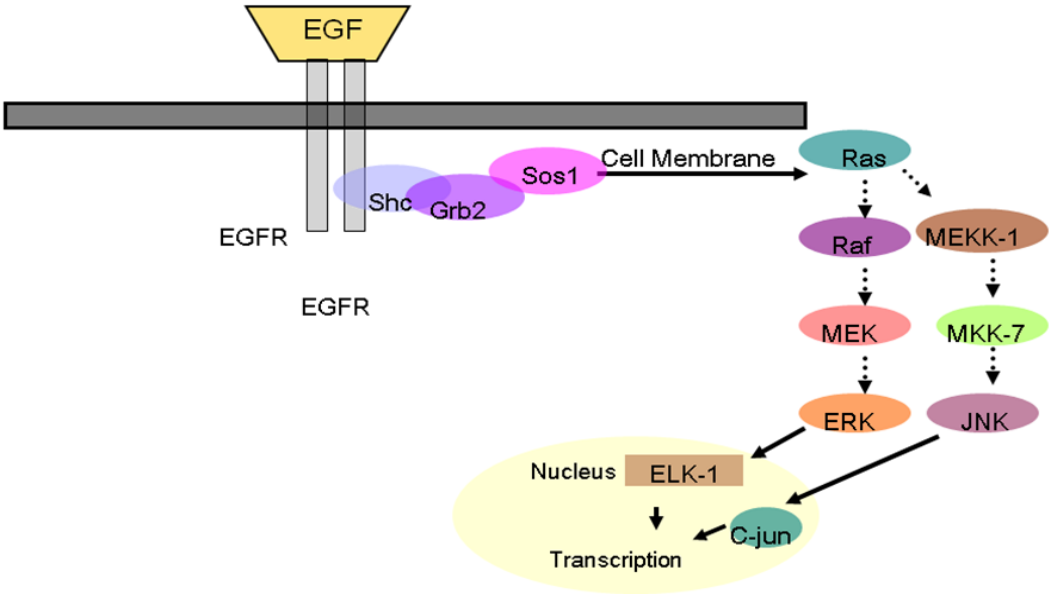

Figure 1. EGFR signaling pathway.

Diverse stimuli may have common effects on mucus gene expression and epithelial cell differentiation. Several EGFR ligands are expressed by airway epithelial cells and appear to serve as the final common pathway to epithelial remodeling resulting from a variety of stimuli (133). For example, neutrophil elastase releases transforming growth factor (TGF)-α from the epithelial cell membrane (162). TGF-α is one of several ligands for the EGFR, specifically ErbB1, that have been demonstrated to be expressed by the epithelium. More recently, it has been reported that multiple TLRs (TLRs 2-6) expressed by the epithelium may lead to activation of the EGFR and induction of airway epithelial cell synthesis and release of IL-8, a neutrophil chemoattractant, and of vascular

endothelial growth factor, a pro-angiogenic factor (98). The pathway involves the activation of NADPH oxidase and the generation of reactive oxygen species. Subsequent activation of TNF-α converting enzyme (TACE) leads to the liberation of TGF-α from the epithelium and ligation of the EGFR (97). TLR2 activation by lipotechoic acid leads to ADAM-10 induced cleavage of heparin binding-EGF, another EGFR ligand. Amphiregulin has also been shown to be released in response to TNFα (33). TLR4 activation leads to mucus gene expression (MUC2) in human epithelial cells via NF-κB activation. NF-κB activation has been described in the mouse airway within 30 minutes of ovalbumin challenge (149).

IL-13 is one of the important Th2 cytokines that triggers the differentiation of goblet cells from epithelial cells (34). It may act on the epithelium in several ways. Wounding of the epithelium grown as a monolayer in culture triggers the epithelial synthesis of IL-13, which subsequently leads to activation of the EGFR through the release of HB-EGF from the epithelial cell membrane (3). Furthermore, IL-4 and IL-13 also induce TGF-α release by epithelial cells that, via autocrine signaling, results in mucous metaplasia (116). IL-4 has been demonstrated to reduce apoptosis of epithelial cells (34). IL-13 causes the release of cysteinyl leukotrienes (cys-LTs) within the airways which in turn may cause transactivation of the EGFR (152). Eosinophils and mast cells are potential sources of cys-LTs (114; 188). Oxygen radicals may be involved in the process of transactivation of the EGFR by cys-LTs. The EGFR mediates cys-LT induced airway epithelial remodeling in the mouse (178) and it appears that this axis is the predominant pathway by which MUC genes are upregulated and that epithelial proliferation is triggered in response to these mediators.

Inhalation of aeroallergen, specifically ovalbumin, leads to the activation of the epithelium. In the sensitized mouse rapid activation of NFκB follows inhalation of allergen. This master transcription factor signals the expression of a host of pro-inflammatory molecules. Among these molecules are fibrogenic cytokines such as transforming growth factor (TGF)-β that may have effects on the underlying tissues (92). Subepithelial deposition of collagen, as mentioned

above, is typical of airway remodeling in asthma and it has been related to the severity of disease. The source of the altered matrix proteins (collagen I and III, tenascin etc.) is not clear although it is quite possible that epithelial synthesis of fibrogenic factors may be important in its formation. The role of the airway epithelium in remodeling goes beyond the histological changes that it undergoes; the epithelium appears to have a key role in determining the remodeling of other tissues in the airway, such as the SM and the connective tissue, through synthesis of growth and fibrogenic factors.

1.7.3 **Airway remodeling-airway smooth muscle**

ASM contraction

ASM, unlike skeletal muscle has no troponin, a regulatory protein activated by calcium (Ca^{2+}) ions (1). Instead, ASM has calmodulin, which interacts with four Ca^{2+} ions and activates cross-bridge cycling (1). The Ca^{2+} -calmodulin complex then joins with and activates myosin kinase, a phosphorylating enzyme (1). Myosin light chain kinase phosphorylates one of the light chains of each myosin head, called the regulatory chain, allowing the head to bind with an actin filament, leading to the actin-myosin interaction (1). This interaction results in movement of the actin filament and causes the contraction of muscle (1). To terminate contraction, myosin phosphatase is required to dephosphorylate the regulatory light chain of the myosin head and is also a limiting factor, determing the time required for the relaxation of muscle (1).

ASM phenotype

Cytokines, chemokines, and growth factors from inflammatory and structural cells are responsible for contributing to airway remodeling and thus, possibly affecting ASM phenotype. The phenotypes of smooth muscle commonly include a more sessile, contractile phenotype varying into a more motile, proliferative/synthetic phenotype.

These phenotypes tend to have reciprocal relationships, so that proliferative cells tend to lose their contractile proteins and *vice versa*. There is

some evidence that these phenotypes which have been best characterised *in vitro* also occur *in vivo*. Moir et al. as well as Labonté et al. noted persistent changes in contractile protein content in Brown Norway rats exposed to ovalbumin (105; 129). It is suggested that when SM is the contractile phenotype *in vitro*, it does not divide when challenged with serum mitogens but can undergo a change of phenotype to a synthetic state in which division can be stimulated (31). Thus, the phenotype of SM and its contractile properties are important to gain insight into the consequences of airway remodeling for airway hyperresponsiveness.

There is abundant evidence in the literature that ASM mass is increased in asthma. In 1969, one of the first systematic quantifications of the changes in ASM was published by Dunnill and colleagues (42). Many studies have subsequently confirmed the increase in ASM mass (26; 103; 145; 163; 169; 190) and most suggest that an increase in ASM cell number, hyperplasia, is responsible (44; 63; 65; 88; 153; 169; 190). Other proposed mechanisms for increased ASM mass include an increase in cell size, hypertrophy (13; 44; 153), migration of ASM or subepithelial myofibroblasts (13), circulating fibrocytes (160) or local mesenchymal stem cells (165) and lastly, epithelial mesenchymal transition (193).

Mechanisms pertaining to hyperplasia and hypertrophy are quite different. It has been shown that the protein endothelin and TGF-ß may induce hypertrophy *in vitro* (55; 121). They are both potential contributors to the hypertrophy observed in some asthmatic subjects (44). The severity and phenotype of asthma may be determining factors of whether hyperplasia or hypertrophy is the contributing factor to increased SM**.**

An excess of proliferating ASM cells has recently been reported for human tissue (63) using PCNA or Ki67 as markers; an attempt to document an excess of proliferating cells in the airway tissues using cyclin D expression did not find any excess of proliferating cells in the ASM but only in the epithelium and subepithelial space (186). Additionally, the balance between decreased apoptosis of SM cells in conjunction with hyperplasia may be an important balance in regulating increased SM mass (151).

It has also been proposed that an altered calcium homeostasis (increased calcium homeostasis) contributed to excessive ASM proliferation in severe asthma via an increased mitochondrial biogenesis (171).

An alternative mechanism for the increased SM mass involves subepithelial myofibroblasts, which are intermediate in phenotype between fibroblasts and myocytes, which hypothetically may proliferate and migrate to join the SM bundles (88; 160). Such a mechanism would require appropriate chemoattractant stimuli from the ASM itself for new muscle to migrate to join the bundles that are present by default. Such a notion is perhaps favored by the finding that ASM grows towards the epithelium in asthma (13). However there is as yet, no evidence that this is an asymmetrical pattern of ASM growth.

An increase in ASM in the central airways has been found in association with severe asthma (26) whereas the increase in peripheral ASM mass was comparable in fatal and non-fatal asthma cases (26). Bronchoscopic biopsies from proximal airways also demonstrate an increase in ASM as a function of the severity of asthma (145). The site of ASM growth may have an important bearing on the behaviour of asthma if indeed the excess ASM is responsible for airway hyperresponsiveness (AHR).

The mechanisms of ASM remodeling are less well explored. Cysteinyl-leukotrienes (cys-LTs) (66; 68; 178; 185) and endothelin have been implicated in murine and rat models *in vivo* undergoing repeated allergen challenges (158). Their precise place in the myriad of events taking place has not been clearly established. Upstream pathways involving cytokines such as IL-4 and IL-13 have been shown to be important for ASM remodeling in a chronic murine model (112). IL-13 upregulates cys-LT1 receptors on human ASM (46) and could conceivably do the same in other species, providing a link between this cytokine and remodeling. IL-13 does not cause proliferation of ASM and on the contrary has recently been shown to inhibit proliferationwithout modulating contractile phenotype in culture (154) and so is likely to have an action on remodeling that is indirect *in vivo*, perhaps through release of EGFR ligand(s) from epithelium (3). It also causes the release of cys-LTs when administered exogenously (131).

However, since IL-4 and IL-13 are upstream in the immune response leading to allergic airway responses there may be indirect mechanisms by which they are contributing. Recent *in vitro* data do suggest however that both IL-4 and IL-13 synergize with fibroblast growth factor (FGF)-2 to induce human bronchial SM cell proliferation (18). This is in contrast to data that demonstrated IL-4 reduced the mitogenic effect of bronchial SM growth factors, including FGF-2 (64). TGF-β also up-regulates the cys-LT1R (46). Mice deficient in the transcription factor, T-bet, which regulates expression of the Th1 cytokine IFN-γ and promotes Th1 type responses, spontaneously develop an asthmatic phenotype including an increase in ASM cells and/or myofibroblasts (49). The lungs of these mice contain activated memory $CD4^+$ T cells that express high levels of Th2 cytokines (IL-4, IL-5, and IL-13) as well as TGF-β (49). In contrast to effects on airway epithelial differentiation and function the role of the EGFR in mediating ASM remodeling is largely unexplored. However, coexpression of the EGFR ligand, amphiregulin, with the allergy-inducing Th2 cytokine pattern raised the intriguing possibility that Th2 cells contribute to both tissue hypertrophy and allergic mechanisms that cause asthma (196).

In conclusion, EGFR ligands are certainly mitogenic for ASM and interact with cys-LTs in promoting mitogenic effects (141; 178).

1.7.4 **Airway remodeling: subepithelial fibrosis**

Fibrosis, another prominent feature of airway remodeling, results from an increased deposition of extracellular matrix, particularly collagen types I and III, fibronectin, and proteoglycans. Mediators including transforming growth factor β (TGFβ) and interleukin-11 contribute to the increased extracellular matrix deposition (29). The composition of the matrix may have functional consequences by altering physical properties of the airway wall (19; 122) and can also influence SM proliferation (15; 71; 87).

Extracellular matrix

Extracellular matrix (ECM) changes have been documented in human asthmatics as well as in murine and rat models of allergic asthma (140; 146). The ECM serves to promote cellular adhesion and enhance cell mobility. Normally, proteins such as collagen and fibronectin provide the substratum for the adherence of cells. In the Brown Norway rat, the amount of collagen is increased by allergen challenge in the large airways. Fibronectin is similarly increased in the airways of rats and mice repeatedly exposed to allergen (127; 140). Proteoglycan and/or collagen deposition is increased in the airway walls of mice chronically exposed to ovalbumin (16; 111). The ECM may influence various functions of ASM, including proliferation via an autocrine mechanism (87). Johnson et al. have previously reported that ASM cells from asthmatics patients proliferate faster than those from nonasthmatic patients (88). They support the notion that the altered profile of ECM protein components including increased perlecan and collagen I, and decreased amounts of collagen IV, laminin α1, and chondroitin sulfate, and not the soluble mediators released from the ASM, influence the proliferation of asthmatic ASM (87). Some of the changes in matrix proteins may serve to limit the over-growth of ASM; *in vitro* data suggest that the proteoglycan decorin plays a role in decreasing ASM proliferation and increasing ASM apoptosis (37). This effect was shown to be specific to decorin (37).

Fibroblasts

Fibroblasts are stimulated by IL-13, an epithelial and T cell cytokine, to synthesize collagen. The chemotactic ability of fibroblasts is increased by TGF-β (148). Interestingly fibroblasts isolated from asthmatic subjects and placed in culture retain a capacity for enhanced collagen synthesis. IL-4 has also been shown to induce human lung fibroblast differentiation into myofibroblasts and to stimulate collagen III secretion *in vitro* (10). The pro-angiogenic cytokine, platelet-derived growth factor (PDGF) induces fibroblasts to produce collagen as well (113).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of multifunctional enzymes which breakdown the extracellular matrix, and have a range of actions that may influence tissue remodeling. The important role of ADAM-17 (TACE) was addressed earlier in regards to the release of EGFR ligands from the cell membrane. However, MMPs also cleave matrix proteins and may make space within the tissues for cells to migrate as well as reveal cryptic sites on matrix molecules with chemoattractant properties, in turn inducing cell migration. ADAM-33 was identified as a candidate gene related to asthma and it was postulated that it might be involved in airway hyperresponsiveness (174). However an ADAM-33 deficient mouse has not demonstrated any significant lung phenotype although responses of the deficient animals to prolonged allergen exposure have not been reported (32).

The ratio of matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 (TIMP-1) has been correlated with FEV_1 (percentage predicted) (181).

Myofibroblasts

Myofibroblasts are cells involved in the alterations in the ECM. The myofibroblast has both synthetic and contractile phenotypes. Myofibroblasts have been shown to be increased in the collagen layer of the asthmatic airway wall (20). Gizycki and colleagues (54) have shown that, in asthmatic patients, allergen inhalation can result in an influx of myofibroblast-like cells into the airway wall 24 hours post-challenge. The precise nature of the myofibroblasts is, however, somewhat controversial. Whether this cell represents a dedifferentiated smooth muscle cell or fibroblast, or a separate cell type entirely, is not clear.

Myofibroblasts have been noted in increased numbers in the subepithelial compartment of the airway wall. It is postulated that they may result from epithelial-mesenchymal transition (EMT), a phenomenon that has been demonstrated to occur in culture under the influence of TGF-β (73). EMT is a process by which epithelial cells acquire mesenchymal, fibroblast-like properties

and they demonstrate reduced intercellular adhesion and increased motility. TGF-β promotes the differentiation of myofibroblasts from fibroblasts (62). Myofibroblasts have attracted a great deal of attention as they may be potential sources of matrix protein. They localize to areas of collagen deposition below the epithelium (160). Several studies have reported an increase in these cells in asthma (54; 91; 186). A marked increase in myofibroblasts occurs following allergen challenge of human asthmatic subjects (91). There is no evidence thus far that migration of these cells to join smooth muscle bundles actually does occur in the asthmatic airway. Recently, it has been documented that cultured myofibroblasts from asthmatics demonstrate reduced proliferative responses (186). However, they display increased cytokine production and this may contribute to persistent inflammation in asthma (186).

1.7.5 ***MicroRNA-regulation of ASM***

MicroRNAs are important candidates for regulating smooth muscle. MicroRNAs are a class of non-coding RNAs, usually ~ 20-25 nucleotides in length, which have been reported to play a role in the proliferation/differentiation balances during tumorigenesis and organ development (24; 94; 199). MicroRNAs have been implicated in inhibiting the target messenger RNAs. MicroRNAs do so by repressing translation or reducing the stability of mRNA. They may also be responsible for activating mRNA translation under some cellular conditions (179). There has been a lot of interest generated in the realm of microRNAs and their potential roles at the post-transcriptional level in cells. miR-29, a regulator of ECM and fibrosis, associated with vascular smooth muscle cells and conducting airways, has been implicated in airway remodeling (104). Similarly, miR-25 has been implicated in ASM regulation as well. Both miR-29 and miR-25 target Kruppel-like factor 4 (Klf4) (99; 104). Furthermore, the article by Cordes, K.R. et al. (2009) demonstrates that miR145 can direct the fate of vascular smooth muscle cells (35). With the support of miR-143, miR145 can regulate the contractile versus proliferative states of smooth muscle cells (35). This concept in itself is unique. The miR-145 and miR-143 were shown to target a number of

transcription factors including Klf4, myocardin and Elk-1 and thus, promoting differentiation and suppressing proliferation of smooth muscle cells (35). The importance here lies in the therapeutic implications of modulating the lowered levels of these particular miRNAs normally found in injured or proliferative states and restoring them to normalcy.

1.7.6 ***Airway smooth muscle-immune effector cell crosstalk and remodeling***

The T cell and the mast cell (MC) have been described to be present in the ASM layer in asthmatic subjects (11; 22). Mast cells, which are known to be involved in immediate hypersensitivity and late phase inflammation, are triggered by cross-linking of high-affinity IgE receptor, FcεRI. Several mast cell-derived mediators including tryptase, histamine, and cytokines such as TNF-α have also been implicated in airway remodeling (136). Tryptase, for example, contributes to airway remodeling by stimulating proliferation of ASM cells (14). In remodeling in asthma, ASM stimulated by Th2 cytokines, IL-4 and IL-13, is chemotactic for mast cells (166). Non-asthmatic ASM releases a mediator(s) that inhibits migration of mast cells towards stimulated ASM in asthmatics (166). CXCR3 is the most abundantly expressed chemokine receptor on human lung mast cells in ASM in asthma (21). It has been found to be expressed by 100% of these mast cells, compared with 47% of mast cells in the submucosa (21). Human lung mast cell migration has been found to be induced by ASM cultures, predominantly through activation of CXCR3 (21). Furthermore, ASM and fibroblasts express functional CCR7, and that both ASM and mast cell-derived CCL19 mediate ASM migration in asthma via activation of CCR7, providing a novel chemotactic pathway for the recruitment of ASM or ASM progenitors to the asthmatic ASM compartment (90).

The T cell has been implicated in ASM growth *in vitro* through direct contact mechanisms (108; 151). Adhesion molecules such as CD44 have been implicated in the T cell-induced growth of ASM. Contact dependent mechanisms might also result in the release of soluble mediators that are then responsible for ASM proliferation. Activated Th2 CD4+ cells release amphiregulin (197), an

EGFR (ErbB1) ligand, providing a possible mechanism by which the T cells in asthma may mediate ASM remodeling. Mast cells also synthesize amphiregulin (100). Dendritic cells produce cys-LTs and up-regulate LTC_4 synthase and 5-lipoxygenase when incubated with antigen. The cys-LTs are, in turn, involved in Th2 cell differentiation (117). It seems likely that Th2 cells will therefore be more pro-proliferative.

1.7.7 **Airway remodeling: angiogenesis**

Histological analysis of bronchial biopsies indicates an increased number of vessels in asthmatic subjects compared to controls (27; 115). The number of vessels per area tissue has also been reported to be higher in mild asthmatics (115; 159). Vessels may increase in number via proliferation of vessel branches or elongation of the existing vessels while microvascular enlargements can result from endothelial cell proliferation (23). The number of vessels has also been correlated with the severity of disease (139; 159).

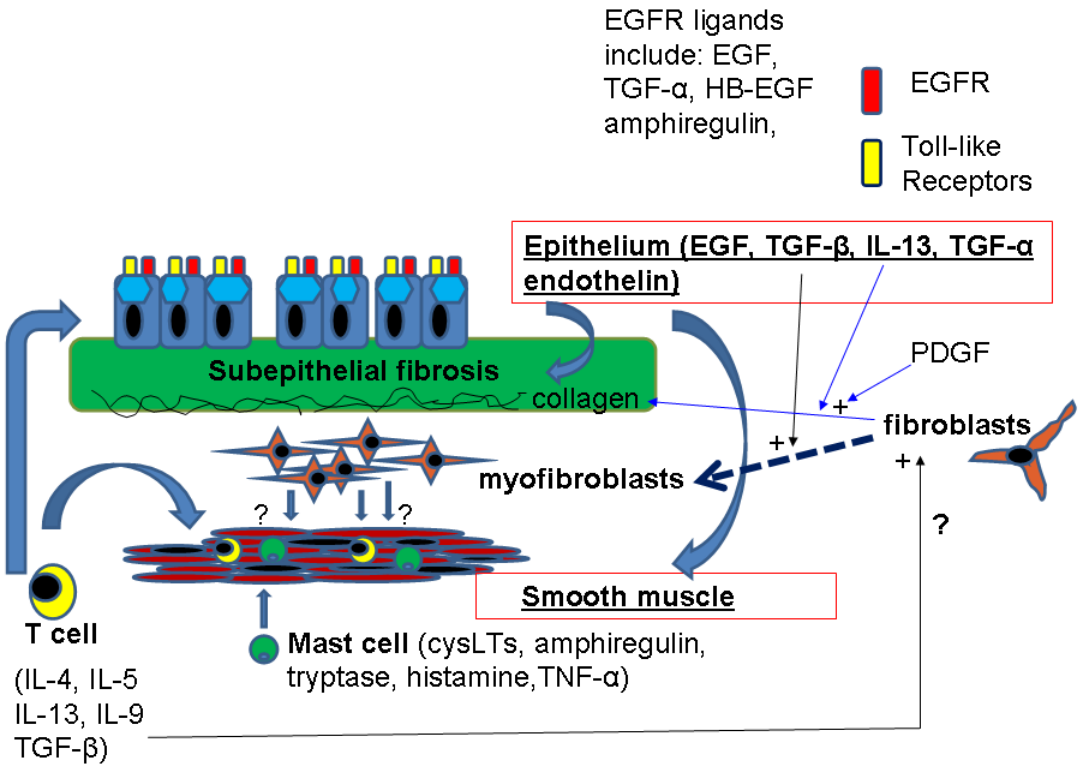

Figure 2. Summary of some key features of airway remodeling.

1.7.8 **Physiologic consequences of airway remodeling**

While the aforementioned structural changes may contribute to AHR and the decline of lung function, the exact relationship between airway remodeling and AHR has not been entirely understood over the years of research. The forced expiratory volume in the first second of a maximal expiratory effort (FEV_1) is the most commonly used parameter to assess airflow limitation in the airway. Maximal flow decreases with decreasing airway radius, which explains why individuals with narrowed or obstructed airways, as observed in asthma, have a reduced FEV_1. Given that the relationship of airway remodeling and AHR was still of interest in the scientific community when we began our studies, a basic, underlying premise for our investigative work focused on further contributing to the present knowledge on the relationship between airway remodeling, primarily ASM growth and goblet cell hyperplasia, to AHR.

The physiologic consequences of airway remodeling include airway hyperresponsiveness (AHR) from ASM growth and mucus hypersecretion from gland and goblet cell hyperplasia. Benayoun and colleagues (13) studied the varying severity of asthma amongst subjects. Using endobronchial biopsies, they sampled ASM, mucus glands, subepithelial basement membrane and found that severe asthmatics were distinguished by larger numbers of fibroblasts, an increased mucus gland size, an increased ASM area, and increased cell size, compared with those of less severe asthmatics, controls, and patients with chronic obstructive pulmonary disease (13). Moreover, airflow obstruction was inversely correlated with fibroblast number and ASM cell size (13). This is the first study linking changes in airway smooth muscle with indices of altered physiology.

The fibroblast is an important cell responsible for the synthesis and secretion of ECM proteins. The process by which the number of fibroblasts increases in asthma is poorly understood, but epithelial–mesenchymal transition (EMT) may play a significant role. In addition to the potential effect of increased SM mass on airway constriction, ASM cells are known to have synthetic properties and capacity to affect extracellular matrix (ECM), especially that in the region of the SM layer (86). Hence, alterations in ASM may also affect airway function via an effect on matrix deposition.

Hyperplasia of the epithelium and goblet cell metaplasia, mucus gland hypertrophy and hyperplasia, subepithelial fibrosis and airway smooth muscle hyperplasia have been perhaps the most studied airway tissue alterations. The physiological consequences of these changes have been debated in the literature but it seems probable that goblet cell metaplasia and mucus gland hypertrophy/hyperplasia cause mucus hypersecretion, that subepithelial fibrosis causes loss of airway distensibility and that airway smooth muscle hyperplasia contributes to airway hyperresponsiveness. Loss of airway distensibility has the potential to lead to airway hyperresponsiveness also through reduction in the strain transmitted to the ASM by cyclical breathing movements, which is under normal circumstances a potent bronchodilator (51; 56). Lambert has argued that an increase in ASM mass provides greater tension in the asthmatic airway and is

sufficient to account for airway hyperresponsiveness (106). However, it seems likely that both loss of airway distensibility and increase in ASM both contribute to AHR. It is possible that ASM hyperplasia is associated with a loss of contractile proteins, for a period of time after hyperplasia has occurred (30; 105; 129). Recovery of contractile phenotype is likely.

On the other hand, human asthmatic studies using non-invasive high-resolution computerized tomography scanning to assess airway wall thickening indicate that in asthma, increased airway wall thickening is coupled with a reduced reactivity to methacholine (134). Paré proposes that the inconsistency between studies for asthma that use mathematical modelling versus those that use computerized tomography to evaluate airway wall thickness and airway responsiveness, is that the former are based on airway geometry, not fully accounting for the potential contribution of airway wall thickening to stiffness and other mechanical properties of the airway (142). A great deal of work has been carried out to model effects of a thickened airway wall on airway mechanics. Two factors require careful consideration. The first consideration is the effect of a thickened airway on airway resistance by virtue of encroachment on the airway lumen. In laminar flow conditions, resistance is proportional to the inverse of the radius to the fourth power ($1/r^4$). Therefore, even a relatively small decrease in the diameter of the airway lumen will have a great impact on the amount of airflow resistance. The second consideration is the effect of a thickened airway wall on induced airway narrowing such as occurs during a methacholine challenge used to measure airway responsiveness.

Moreno and et al (130; 189) generated a model that allowed the study of the effects of airway wall thickening on both airway resistance and induced airway narrowing. Fundamentally, this model predicts that, as there is airway wall thickening, the amount of luminal narrowing as a consequence of ASM shortening will be greatly enhanced. Interestingly, the effect on baseline airway resistance may not be changed substantially but the effect on the resistance of the constricted airway would be great (144). Furthermore, due to the airway wall thickening in asthmatic airways, for complete airway closure, less ASM

shortening is required (81). The same investigative group, using this modeling approach and morphometric measurements taken from asthmatic patients, posited that increased SM mass is the primary determinant of the increased airway resistance in response to constrictor stimuli in asthmatic subjects (106). Hence, remodeling and thickening of the subepithelial and smooth muscle layer, such as has been demonstrated in asthmatic airways, would be expected to have important consequences in terms of the physiologic function of the airways.

James, on the basis of observed changes in airway wall thickness, proposed that in small membranous airways as compared to cartilaginous airways the same degree of smooth muscle shortening resulted in approximately tenfold greater increases in airway resistance (81). Furthermore, Lambert et al. with the aid of a computer model was able to differentiate the relative contributions of increases in the submucosal, smooth muscle, and adventitial airway subdivisions in the context of airway constriction (106). They identified different airway narrowing mechanisms which would be determined by the airway wall region (106). Changes in the submucosa caused an increased luminal occlusion for a given amount of muscle shortening, while increases in the adventitia contributed to airway constriction via decreasing the tethering effects of the lung parenchyma. Altered airway structure, including airway wall thickening, is observable both in the large and small airways in severe (fatal) asthmatic cases, but only in small airways of mild cases (26).

It has been more difficult to ascribe pathophysiological importance to matrix protein deposition. Altered stress-strain relationships may lead to a reduced extent of ASM stretch caused by tidal breathing and following deep breaths. It is anticipated that this may enhance the pre-disposition of the airways to undergo excessive airway narrowing in the absence of the potent bronchodilating influence of cyclical airway stretching. Altered matrix proteins may also have an influence on the predisposition of tissues such as ASM to undergo proliferation or to resist proliferative stimuli under some circumstances (17; 37; 71). The tendency to be in a secretory/proliferative state rather than the more usual contractile phenotype is likely related to the matrix composition but

there are few data on this phenomenon *in vivo* despite its extensive exploration *in vitro*. The complex molecular remodeling of the airway epithelium is responsible for many of the manifestations of asthma (3; 73; 92; 147), in particular the pattern of inflammation.

Smooth muscle can only narrow the lumen of the airway it surrounds if it can generate enough force to overcome the forces opposing it. Principal opposition is provided by the elastic recoil of the airway wall and the outward tethering forces exerted by the parenchyma within which the airway is embedded. Airway remodeling could affect stiffness and hence the balance of forces that determine how much airway narrowing occurs when the smooth muscle is activated. It remains controversial as to whether remodeling is likely to enhance or reduce airways responsiveness.

The outward tethering forces exerted by the parenchymal attachments to the outside of the airway wall have an enormous influence on the ability of the smooth muscle to shorten. Attachments transmit transpulmonary pressure across the airway wall, and it is this pressure that opposes shortening of the airway smooth muscle. Consequently, increasing lung volume causes a dramatic decrease in airways responsiveness (9; 39).

Airways responsiveness is not only determined by a balance of forces. Geometric factors also contribute. For example, when ASM shortens around the airway, it compresses the tissue making up the inner part of the airway wall, notably the mucosal lining. Volume of this lining is thought to be preserved during narrowing, so it necessarily becomes thicker. This causes the lumen to be reduced much more rapidly than would be the case if no mucosal lining were present (130). Accordingly, any situation in which the mucosa becomes thickened, such as during inflammation, caused by an allergic reaction, can enhance luminal narrowing even when the strength of the SM and elastic forces opposing it are completely normal. Accumulations of airway secretions can have a similar effect.

These and other factors individually have the potential effect airway responsiveness to a substantial dgree, so it is tempting to speculate about which

might be the most potent and likely to be responsible for a disease such as asthma. In reality, these factors probably co-exist. Indeed, they can act together in a synergistic fashion, so it is likely that multiple factors are at play.

1.9 **Assessment of respiratory mechanics**

Total respiratory system resistance (R_L) and elastance (E_L) are commonly used to quantify the response of the lungs to allergen challenge (83). W hen a SM bronchoconstrictor such as methacholine is administered to the lungs via aerosolization or an intravenous injection, the lungs undergo a bronchoconstrictive response (83). Durring bronchoconstriction narrowing of the conducting airways due to contraction of SM in the airway wall is the main occurrence (83). Dose-response relationships are usually graphed, reflecting R_L and E_L after exposure to agonist (83). These entail two important points (83):

1) Dose of agonist required to elicit a response significantly above baseline, which gives a measure of *sensitivity*.
2) Response elicited by a dose of agonist which reflects maximal *responsiveness* or *maximal reactivity*.

Animals that display asthmatic features may tend to be hypersensitive and/or hyperresponsive compared to control animals.
In normal subjects, extremely high concentrations of bronchoconstricting substances can be inhaled with only mild-to-moderate airway narrowing (83).

Resistance and elastance are calculated by the single compartment model:
P_{tr} = resistance * flow + elastance *Volume +P_o

P_{tr}= tracheal pressure
Volume= tracheal volume
P_o is a fitting constant

The variables of the model are P, V, flow and its parameters are R and E. P_{tr} and V_{tr} calculated from displaced volume and cylinder pressure. Flow is calculated by

differentiating volume. The resistance and elastance terms are fit using multiple linear regression with P_{tr} as the dependent variable and flow and V_{tr} as the independent variables (83).

For the forced oscillation technique, P_{tr} and volume waveforms are used (83). First input impedance is calculated – this results in 2 graphs, resistance and reactance versus the frequencies present in the primewave signal used (83). Then the parameters from the constant phase model are calculated using the input impedance data (83).

Constant phase model

$$\text{Zin } (f) = R + i2\pi fI + \frac{G_t - iH_t}{(2\pi f)^\alpha}$$

.

The constant phase model allows the partitioning of the lung into central and more peripheral lung compartments (60). We are able to acquire data for R (Newtonian resistance), which reflects resistive changes occurring in the conducting airways while changes in G (tissue damping) and H (tissue elastance) reflect changes in the intrinsic rheological properties of tissues (60; 83). If a fraction of the lung becomes closed off from the airway opening, as a result of either airway closure of alveolar collapse, then both G and H will increase by the same fraction (83). Pure decruitment thus preserves η (η=G/H). On the other hand, if the lung becomes heterogeneous than G will very likely increase proportionately more than H(83).

2.0 **Airway remodeling: Is it all bad?**

Modification of the viscoeleastic properties of the airway wall either at the level of the submucosa, the SM, or the adventitia, may change the load against which the ASM mass must shorten. Furthermore, increased tissue resistance and/or elastance would make shortening of the ASM more difficult. Changes in the reticular basement membrane could change the folding tendencies of the bronchial mucosa and subsequently, a decrease in airway lumen with induced

constriction. Finally, alterations in the viscoelastic properties of the matrix could have an impact on the response of the ASM to volume perturbations, such as that observed with a deep breath. In subjects with mild asthma, a deep breath can ameliorate ASM constriction but in severe asthma, a deep breath in itself can have a bronchoconstricting effect (52). A matrix with altered viscoeleastic properties could also have an impact on the transmission of strain to the SM cell. Possibly one of the key differences between mild and more severe asthmatics is in the ability of the matrix to remodel in the appropriate way. All these considerations are important as we review findings between asthmatics of varying severity versus controls.

2.1 **Corticosteroids**

Corticosteroids are first line anti-inflammatory therapeutic agents. The inhibition of airway remodeling by corticosteroid administration could potentially improve corresponding airway mechanics. However, results are conflicting. First, there are discrepant data on the ability of inhaled corticosteroids to reverse airway remodeling. Jeffrey et al (84) demonstrated changes in inflammatory cells in the airway wall of asthmatics who received inhaled budesonide but they reported no difference in thickening of the reticular basement membrane. Other investigators have shown steroid effects on remodeling. In various studies (138; 172) decreases in tenascin, collagen type III, and basement membrane thickness have been described after steroid treatment durations ranging from 4 weeks to 4 months. Differences in type of inhaled steroid, dosage and treatment duration may be responsible for these variations in reports. One notion that is very important to keep in mind is that corticosteroid treatment must be provided before remodeling is established. Vanacker and colleagues investigated this possibiliy in the BN rat model of asthma (176). They showed that inhaled steroids (fluticasone) affected subsequent allergen-induced airway remodeling only if steroids were administered concurrently with the allergic stimulus (176). Henderson et al., showed in a murine model of asthma, that dexamethasone administered after established airway remodeling could not inhibit remodeling on

its own (66). Steroids delivered once airway remodeling was established had no effect.

Given that steroids can modulate airway remodeling, the next question is whether changes in the structure are accompanied by the expected changes in physiologic function. There are discrepant data regarding this question. Whereas Olivieri et al. (138) found changes in both the basement membrane thickness and bronchial responsiveness (as measured by PC20), Trigg et al. (172) found no differences in FEV1 or PC20 despite decreases in their index of remodeling (collagen type III deposition in the lamina reticularis). In a study conducted in the rat model of asthma (175), differences have been reported in both fibronectin deposition and airways responsiveness. Interestingly, none of these studies directly correlate changes in airway remodeling and function. In one study, an attempt was made to define steroid-induced changes in vascularity (139). Asthmatic patients receiving beclomethasone were shown to have reduced numbers of vessels in the lamina propria compared with steroid-free asthmatic patients. Furthermore, the authors attempted to correlate numbers of vessels with airway responsiveness and bronchodilator response. They found an inverse relationship between steroids and salbutamol-induced bronchodilation but the r^2 values were of the order of 0.14 and 0.24 respectively.

Corticosteroids have been shown to enhance SM relaxation by increasing adenylate cyclase activity (126), reducing β2 receptor desensitization (119), increasing the number of β2 receptors (118), and the Na+/K+ pump activity(161). Dexamethasone, a corticosteroid, has multiple effects on SM function (70) including the reduction of intracellular calcium (170), uncoupling of H1 histamine receptors (61), and the reduction of muscarinic receptor expression (132). Together, these effects could act in concert to reduce SM contractile activation in response to a variety of stimuli.

Corticosteroids can also reduce SM proliferation (155). Numerous studies have demonstrated that corticosteroids increase the expression of dualspecific phosphatases, especially MKP-1(MAPK phosphatase 1) (89; 107), and by doing so decrease p38 MAPK activity, which is dephosphorylated and

inactivated by MKP-1 (107). It has been previously demonstrated that when p38 MAPK activity is inhibited pharmacologically with, force fluctuation-induced smooth muscle re-lengthening (FFIR) is enhanced (36). Thus, corticosteroids enhance FFIR in contracted ASM and inhibit the p38 MAPK pathway. Others have reported the importance of deep breaths in reversing bronchoconstriction and that this phenomenon which is impaired in asthma is restored by corticosteroid treatment. It has also been suggested FFIR is a mechanism by which deep inspirations protect against bronchoconstriction and that corticosteroids may restore this effect that is impaired in asthma, through inhibition of the p38 MAPK pathway and augmentation of FFIR

The inhibitory actions of corticosteroids are exerted on a variety of inflammatory cells involved in lung diseases. They inhibit mediator and cytokine release as well as chemokine generation from eosinophils (128). Corticosteroids also reduce eosinophil survival by increasing apoptosis, and block their increased survival in the presence of interleukin-5 and granulocyte-macrophage colony stimulating factor (GM-CSF), potentially by activating endonucleases involved in programmed cell death (43). T lymphocytes are an important target of corticosteorids. Although corticosteroids do not appear to have a direct effect on mediator release from lung mast cells, long-term corticosteroid therapy reduces mast cell numbers in tissues. This may be through an inhibitory effect on local synthesis of c-kit ligand, stem cell factor and IL-3 (78). Corticosteroids also inhibit mediator and cytokine secretion by basophils and induce their apoptosis (195). In contrast, corticosteroids increase the survival of neutrophils (198).

At the level of the nucleus, corticosteroids suppress multiple inflammatory genes that are activated in asthmatic airways by reversing histone acetylation of activated inflammatory genes. This mechanism acts by binding of the activated glucocorticoid receptors to coactivators and recruitment of histone deactylases (HDAC2) to the activated transcription complex. In the case of a poor response to corticosteroids observed in patients with severe asthma, a reduction in HDAC2 levels may be at play.

2.2 **Conclusions**

There is a complex web of interaction involving structural and inflammatory cells that may result in structural changes observed in asthmatic airways. Therapeutic interventions in animal models have suggested for the roles of cysteinyl-leukotrienes, endothelin, EGFR and various cytokines involved in remodeling. Mast cells and T cells present in remodeled smooth muscle may also participate in tissue remodeling by mechanisms that are contact-dependent or contact-independent. There are insufficient clinical trials for common asthma medications, corticosteroids being an exception. Corticosteroids although known for their anti-inflammatory effects have limited efficacy against established airway remodeling. Bronchothermoplasty, a technique which uses radiofrequency waves to heat and kill ASM cells in the airway wall, thereby targeting the increased smooth muscle mass, is currently under investigation for treatment. Understanding the basic mechanisms involved in airway remodeling and airway smooth muscle phenotypic modulation in asthma as well as the relationship between the two features and AHR will be helpful in attempting to further address the issue of treatment of this aspect of asthma.

2.2.1 ***Questions addressed in this thesis***

Three major issues were addressed in the thesis. The first question posed was whether the assessment of airway mechanical responses to allergen challenge using the constant phase model could be interpreted in terms of airway narrowing affecting discreet regions within the airway tree. To address this question we used the ovalbumin sensitized and challenged Brown Norway rat in which we assessed respiratory mechanics and compared the changes in mechanical parameters to directly visualized airway narrowing using rapid freezing of the lungs and morphomteric analysis of airway narrowing. The next question posed was whether allergen induced airway hyperresponsiveness affected all-sized airways in the allergen challenged rat and whether the remodeling of the airways, in particular increase in ASM mass and goblet cell differentiation were tightly coupled to AHR. The third question was whether the phenotype of ASM is

modulated during the process of airway remodeling in sensitized Brown Norway rats and whether the dissociation between an increase in ASM mass and the site of AHR could be explained by alterations in the expression levels of contractile proteins. To address these questions we used the BN rat model briefly described next.

2.2.2 ***The Brown Norway rat model***

Animal models provide abundant knowledge concerning the immunological response to inhaled and thus, exogenous antigens and its short-term consequences for airway function. We have used the Brown Norway (BN) rat model (158; 169), a well-established experimental asthma model for our studies. The BN rats used in the studies were imported from Harlan/UK. BN rats are atopic and thus upon exposure to soluble antigensthe T cells potently stimulate B cell production of antibodies, in particular IgE, and subsequently, the rats develop asthma-like symptoms upon allergen exposure. In our *in vivo* model, BN rats were sensitized with ovalbumin (Ova), a protein derived from chicken egg, which acts as an allergen in our experimental asthma model of choice. It is administered *subcutaneously* along with aluminum hydroxide which acts as an adjuvant. Additionally, another adjuvant administered intraperitoneally, concurrently, is heat-inactivated *Bordetella pertussis*. The BN rat is among the best-characterized models for our studies of the EAR and the asthmatic phenotype after multiple allergen challenges.

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Chapter 2

Site of allergic airway narrowing and the influence of exogenous surfactant

2.3 **Prologue**

The constant phase model introduced in the early 1990s by Hantos et al. (1992) allows the attribution of changes in the mechanical properties of the lungs to the conducting airways (R_N parameter) and the peripheral lung compartments (G and H parameters). We wished to assess the site of airway narrowing during the early allergic response (EAR) with the constant phase model and to compare the changes in mechanical parameters to the assessment of airway narrowing by morphometry. We then explored possible determinants of the EAR; mast cell and airway smooth muscle mass across varying airway sizes before considering the importance of surface tension in the modulation of EAR. We used surfactant administration as a probe of the system, anticipating salutary effects on peripheral airway narrowing. We used a commercially available surfactant, lacking surfactant proteins A and D, on the pulmonary mechanical changes, associated morphometry, mast cell activation and influence on mast cell mediators associated with the EAR as well as serotonin-induced responses.

2.4 **Abstract**

Background: The parameters (R_N, G and H) of the constant phase model of respiratory mechanics provide information concerning the site of altered mechanical properties of the lung. The aim of this study was to compare the site of allergic narrowing implied from the constant phase model to a direct assessment by morphometry.

Methods: We induced airway narrowing by ovalbumin sensitization and challenge and we tested the effects of a natural surfactant lacking surfactant proteins A and D (Infasurf®) on airway responses. Sensitized, mechanically ventilated Brown Norway rats underwent an aerosol challenge with 5% ovalbumin or vehicle. Other animals received nebulized surfactant prior to challenge. Three or 20 minutes after ovalbumin challenge, airway lumen areas were assessed on snap-frozen lungs by morphometry.

Results: At 3 minutes, R_N and G detected large, whereas at 20 minutes G and H detected small, airway narrowing. Surfactant inhibited R_N at the peak of the early allergic response and ovalbumin-induced increase in cysteinyl leukotrienes and amphiregulin but not mast cell activation *in vitro*.

Conclusion: Allergen challenge triggers large airway narrowing, detected by R_N and G, and subsequent peripheral airway narrowing detected by G and H. Surfactant inhibits airway narrowing and reduces mast cell-derived mediators.

Word count: 198

Key words: airway smooth muscle, amphiregulin, bronchoconstriction, Brown Norway rat, cysteinyl leukotrienes, surface tension

2.5 **Introduction**

The constant phase model of respiratory mechanics has been proposed for the partitioning of pulmonary responses to the airways and tissues (1;2). Changes in mechanics directly attributable to narrowing of the conducting airways is detected by R_N and changes in tissue viscous resistance (tissue viscance or tissue damping) are detected by the G parameter. Tissue damping increases with heterogeneity of bronchoconstriction and hyperinflation (3;4). Hyperinflation and inhomogeneity of ventilation distribution also contribute to increases in tissue elastance (H parameter) (5). Since airway resistance is predominantly contributed by the larger conducting airways, the constant phase model parameters may partition the airway responses into those related to the large airways (R_N) and to the peripheral lung (G and H). The site of airway narrowing following methacholine or allergen-induced bronchoconstriction may be directly assessed by snap-freezing lungs and performing morphometric measurements (6).

Allergen challenge of sensitized subjects may result in early and late responses (1). The early response occurs within minutes after allergen exposure and results from mast cell degranulation and the release of preformed biogenic amines including histamine and serotonin and the *de novo* synthesis of cysteinyl leukotrienes (CysLTs) (7;8). These mediators also cause a vascular leak into the airways (9;10). Microvascular leak of proteins as well as surfactant phospholipid changes such as occur during allergen-induced airway reactions may disrupt surfactant function (11;12) and serve as an additional mechanism for airway narrowing following allergen challenge. Surfactant administration may be beneficial in restoring surfactant activity (13) and we reasoned it would preferentially reduce peripheral airway narrowing following allergen challenge.

The first objective of the current study was to compare the site of airway narrowing as determined by the constant phase model and by morphometry during the early allergic response (EAR). The second objective was to assess the potential modulation of changes in airway mechanics following Ova or serotonin-induced bronchoconstriction by the administration of surfactant. To examine if

surfactant might inhibit the allergic response itself, we also assessed the mast cell mediators, CysLTs and amphiregulin, in bronchoalveolar lavage fluid immediately after the peak of the EAR after Ova challenge. Additionally we examined the effects of surfactant on Ova-triggered mast cell activation *ex vivo*.

2.6 **Methods**

Animals and sensitization to ovalbumin

Male Brown Norway (BN) rats, 6-8 weeks old and weighing 160-180g (SsN substrain) (Harlan/UK), were sensitized subcutaneously with 1mg ovalbumin (Ova) and 100mg aluminum hydroxide dissolved in 1 ml of phosphate buffered saline (PBS) (14). Concurrently, the rats were injected intraperitoneally with $2x10^9$ *Bordetella pertussis* heat-killed bacteria (provided by T. Issekutz, Dalhousie, University, Halifax, NS, Canada) (14). The study protocol was approved by the Animal Care Committee of McGill University.

Measurement of early allergic response (EAR) to ovalbumin challenge

Fourteen days after sensitization, rats were endotracheally intubated under light pentobarbital anesthesia and then administered an aerosol challenge for 1 minute, with either 5% Ova or the PBS control, using an ultrasonic nebulizer. Paralysis was induced with 1mg/kg pancuronium bromide intraperitoneally (Sandoz Canada Inc, QC, Canada). The animals were placed on a heating pad and their body temperature was monitored with a rectal thermometer. The animals were ventilated at a tidal volume of 8 mL/kg, a breathing frequency of 90 breaths/minute and an end-expiratory pressure of 2.5 cmH_2O. Respiratory system mechanics were assessed every 15 seconds using the constant phase model (2) with a small animal ventilator (Flexivent, Scireq, Montréal, QC, Canada) up to 20 minutes after the Ova or PBS challenge.

Effects of surfactant on respiratory mechanical responses to ovalbumin challenge

Sensitized rats were anesthetized, paralyzed and mechanically ventilated as described above. Following a baseline recording of mechanics, the animals were administered an ultrasonic nebulization of 200 μL of a commercially available surfactant (~7mg phospholipids), Infasurf® (ONY, Inc, Amherst, NY) or saline, for 5 minutes. This intervention was immediately followed by an Ova or PBS challenge for 1 minute. The animals had measurements of respiratory mechanics every 15 seconds for the subsequent 20 minutes. Infasurf® contains natural phospholipids, neutral lipids and hydrophobic surfactant associated proteins in 0.9% sodium chloride including surfactant protein (SP)-B (15;16). According to the manufacturer, Infasurf® does not contain surfactant proteins A and D, collectins that have been shown to have immunomodulatory effects (as reviewed in (17-19)).

Assessment of airway narrowing by morphometry

Separate groups of animals underwent sensitization and challenge with Ova as described above but were sacrificed ~3 minutes or at 20 minutes following challenge and the lungs were snap-frozen for morphometric assessment (n=4-7). A constant pressure of 4 cmH_2O was applied during freezing so as to fix the lungs at a lung volume within the range of tidal breathing during fixation (6;20). The chest cavity was rapidly opened and liquid nitrogen was poured covering the lungs (6). The lungs were removed and immersed in Carnoy's solution and stored at -80^{o}C overnight (6). The following day the lungs were processed in a series of modified Carnoy's solutions and the tissue was paraffin-embedded (6).

Histological sections (5 μm) were cut from mid-sagittal and para-hilar regions of the lung and were stained with hematoxylin and eosin. The lumen area was traced for at least 10 airways per animal and the ideal lumen area was calculated from the internal basement perimeter (P_{BM}) on the assumption that the fully dilated airway is a circle. All airways in the whole lung section whose aspect ratio (maximal to minimal internal diameter) was less than 2, were

included in the analyses. The median P_{BM} of the airways studied after a single challenge was 0.89 mm. To assess airway narrowing heterogeneity, the coefficient of variation of the airway lumina was calculated for airways $\geq$0.89 mm and for those airways <0.89 mm. Lung sections from a separate group of Ova-sensitized animals were stained with toluidine blue for the assessment of mast cell distribution (n=8) and smooth muscle (SM) α-actin for airway smooth muscle (ASM) assessment (n=6).

Assessment of cysteinyl leukotriene and amphiregulin concentrations in the bronchoalveolar lavage (BAL) fluid at the peaks of EAR

Separate groups of animals were used to assess the CysLTs (n=5-8/ group) and amphiregulin concentrations (n=7-9/ group) in the BAL fluid following Ova or PBS challenge. Two weeks after sensitization with Ova, the animals were anaesthetized, paralyzed and mechanically ventilated as described above. Saline or surfactant was administered for 5 minutes using an ultrasonic nebulizer followed by either an Ova or PBS challenge. At peak EAR, the animals were sacrificed and underwent BAL. The first 5 mL of BAL fluid was stored at -80^{o}C for further analysis. Amphiregulin levels were assayed using the human Amphiregulin kit (R&D Systems, Minneopolis, MN). For the CysLT measurement, samples were extracted using Sep-Pak cartridges (Waters, Milford, MA) and subsequently analyzed after 8-fold dilution using the CysLT Express EIA Kit (Cayman Chemical Company, MI, USA).

Assessment of serotonin-induced bronchoconstriction in the presence of surfactant

Serotonin (serotonin hydrochloride, 5-HT, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was used to induce the bronchoconstriction. 5-HT (30 mg/mL) was nebulized in non-sensitized, anesthetized, and paralyzed BN rats for 20 seconds (n=8) (Flexivent, Scireq, Montreal, QC, CA) (9). Another group of animals (n=8) was administered nebulized surfactant (Infasurf®, ONY, Inc., NY, US) for 5 minutes prior to the 5-HT administration. Subsequently, the

bronchoconstrictive response was assessed using the constant phase model over 20 minutes.

Assessment of intracellular calcium release in RBL-2H3 mast cell line

The RBL (rat basophilic leukemia)-2H3 mast cell line (American Type Culture Collection, USA) was maintained in Dulbecco's modified Eagle medium (DMEM) with 20% fetal bovine serum and antibiotics (penicillin-streptomycin-amphotericin B). Cells were plated on glass coverslips until they reached confluence. About 4 hours prior to measurements, serum was removed and replaced by PIPES buffer containing 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose and 10 mM piperazine-N-N′-bis (2-ethanesulfonic acid); pH 7.4). For the experiments performed in the absence of external calcium, PIPES–EGTA buffer (1.0 mM EGTA) was used. Thirty minutes prior to each measurement, RBL-2H3 cells were loaded with 5μM Fura-2 AM (Molecular Probes, Eugene, OR), mounted in a Leiden chamber (Medical Systems, Greenville, NY) and viewed under the 40x oil immersion objective on an inverted microscope (Olympus, Tokyo, Japan). Five minutes prior to measurements, 10% of either control (saline) or natural exogenous surfactant was added to the medium. Calcium (Ca^{2+}) signaling was triggered by addition of Ova and serum from Ova-sensitized and challenged rats solution (final concentration: 50 μg/ml Ova, 5% serum) premixed 30 minutes prior to the challenge. Fura-2 was alternatively excited at 340 nm and 380 nm with a PTI Deltascan 1 dual monochromator illuminator (Photon Technology International, Princeton, NJ). Additionally, fluorescent properties of the FURA-2 were verified *in vitro* by performing excitation-scan fluorescence measurements in presence of 0 and 39 μM Ca^{2+} with surfactant or saline. Ratios (340/380) were calculated. R_{max} and R_{min} were calculated in the cell line in response to ionomycin (10 μM) in PIPES and PIPES- EGTA buffer respectively. Fluorescent ratios were converted into Ca^{2+} concentration using the formula of Grynkiewicz (10).

Statistical analysis

Data are presented as mean + SEM. The statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA) as well as SAS v. 9.2 (Cary, NC). Paired means were compared using two tailed t tests. Linear regression was performed using the method of least squares. Comparison of morphometric data between the Ova and PBS-challenged animals was assessed using a linear mixed model with the independent variables size (centered around the P_{BM} median of 0.89 mm), Ova and interaction between Ova and size. At 20 minutes, a term for the square of size was also included. This model adjusts for correlation between airways from the same rats.

For the mechanical parameters of the total respiratory resistance of the surfactant groups, a one-way ANOVA and Newman-Keuls test was performed on data at 3 and 20 minutes. For the corresponding morphometry at 3 minutes, a one-way ANOVA and Bonferroni correction was used. A one-way ANOVA and Newman-Keuls post hoc test was performed on the data for CysLTs and amphiregulin. To assess mechanical parameters of the response in the 5-HT and surfactant + 5-HT groups, two-tailed t-tests were performed for data immediately after the 5-HT administration and at 5, 10, 15 and 20 minutes.

2.7 **Results**

Early allergic response: Constant phase parameters

At 3 minutes after Ova challenge, R_N and G increased significantly compared to baseline ($p<0.05$, n=8-10, figure 1 A, B). R_N declined subsequently so that by 20 minutes after challenge it was no longer significantly increased. However the elevation in G persisted ($p<0.01$, figure 1A, B). H was not increased at 3 minutes but was significantly increased at 20 minutes ($p<0.05$, figure 1C). Normalized parameters for the Ova-challenged animals illustrate the relative progression in the individual parameters over 20 minutes (figure 1D).

At 3 minutes, there was no significant difference in R_N/G compared to baseline suggesting that these parameters are coupled (figure 2A). However, R_N/H was significantly higher than controls at 3 minutes ($p<0.05$, figure 2B). Similarly, G/H or hysteresivity, the ratio of energy dissipation to energy conservation, was higher at 3 minutes in Ova-challenged animals compared to controls ($p<0.05$, figure 2C) but not different amongst groups at 20 minutes.

Airway narrowing: morphometric assessment

Airway narrowing was assessed from the ratio of the lumen area to the ideal lumen, the area corresponding to the airway circumference calculated for a perfect circle. The median airway P_{BM} of all the airways assessed was 0.89 mm. Three minutes after Ova challenge, only the airways $\geq$ 0.89 mm were relatively more constricted than the comparable airways in controls ($p<0.05$, n=5-6, figure 3A). At 20 minutes, the airway narrowing in the Ova-challenged animals was greater in the airways with a P_{BM} <0.89 mm ($p<0.01$) and larger airways were no longer significantly narrowed (n=5-6, figure 3B).

We tested the sensitivity of the statistical significance in differences in larger airway narrowing induced by Ova challenge to the choice of cut-off of airway size. The minimum cut-off which gave significance at 3 minutes was 0.86 mm, corresponding closely to the median airway size, 0.89 mm in P_{BM}. At 3 minutes, in the Ova group the luminal ratio declined as airway size increased as did the difference in luminal ratio between the Ova and PBS groups (figure 3C).

However, at 20 minutes, the difference in luminal ratio between Ova and PBS diminished as airway size increased (figure 3D). At $\geq$ 1.77 P_{BM}, the luminal ratio difference between Ova and PBS groups was no longer significantly different.

Heterogeneity of airway narrowing

Figure 4A is an illustration of hematoxylin and eosin staining in an airway from a PBS and Ova-challenged animal respectively. At 3 minutes after Ova or PBS challenges there were no significant differences in the coefficients of variation of luminal areas amongst groups (n=4-6, figure 4B). In comparison at 20 minutes, the smaller airways (<0.89mm) showed significantly greater heterogeneity of airway narrowing after Ova challenge compared to the controls ($p<0.05$, n=6) while there was a strong trend ($p=0.09$) for the airways with a $P_{BM} \geq 0.89$ mm to also show heterogeneity of airway narrowing compared to their respective controls (figure 4C).

Potential determinants of the distribution of airway narrowing during the EAR

We wished to explore some of the possible determinants of differences in the distribution of airway narrowing from large to small airways. There were no clear differences in the distribution of the mast cells in non-Ova-challenged animals between airways of $P_{BM} \geq 0.89$ mm and <0.89 mm (supplemental figure 1A). ASM mass in Ova-sensitized, non-challenged animals was normalized for the square of the P_{BM} to correct for airway size. There were no differences in the normalized SM as a function of airway size (supplemental figure 1B).

Surfactant effects on the mechanical parameters and on airway narrowing as assessed by morphometry during the EAR

At 3 minutes after challenge there was a significant increase in R_N from baseline in Ova-challenged animals which was decreased by exogenous surfactant administration ($p<0.05$, figure 5A). The G parameter in the Ova-challenged animals increased significantly at 3 minutes ($p<0.05$, figure 5B), while there were no changes in the H parameter (figure 5C). By 20 minutes after Ova challenge

R_N, G and H parameters were not significantly different from baseline values (data not shown).

In the Ova-challenged animals at the peak of EAR, the airways $\geq$ 0.89 mm P_{BM} were significantly bronchoconstricted ($p<0.05$, figure 5D). The morphometric data for the airways $\geq$0.89 mm showed a lesser degree of airway narrowing after Ova challenge in animals pre-treated with surfactant. There were no significant differences in the Ova-challenged animals compared to controls in the airways <0.89 mm (figure 5D). Given the lack of changes in mechanical parameters at 20 minutes, morphometry was not performed at this time point.

Effects of surfactant on inflammatory mediators (cysteinyl leukotrienes and amphiregulin) in the BAL fluid

Assessment of the concentration of total CysLTs from the BAL at the peak of the EAR in the study groups revealed an elevated concentration in the Ova-challenged animals compared to controls ($p<0.05$, n=5-7, figure 6A). This increase in CysLTs was abrogated in animals that received exogenous administration of surfactant ($p<0.05$, n=5-8, figure 6A). Similarly, surfactant prevented the increase in amphiregulin that was induced by Ova-exposure during peak EAR ($p<0.05$, n=7-9, figure 6B).

In vivo assessment of EAR responses with serotonin-mediated bronchoconstriction in the presence of surfactant

Serotonin was administered at a high dose to induce bronchoconstriction with a time course not dissimilar to the EAR. Following serotonin R_N, G and H increased. Surfactant reduced the change in R_N at both the peak of the response, and at 5, 10 and 15 minutes into the response (n=7, $p<0.01$, $p<0.05$, figure 6C). The reduction of G was borderline significant at 5 and 10 minutes (p=0.08 and 0.065 respectively, figure 6D) while H was not significantly altered at any time point (data not shown).

Effects of surfactant on RBL cell activation by Ova

RBL cells were used to model the effects of surfactant on Ova-mediated mast cell activation. First we tested the effects of surfactant on the detection of calcium in a cell-free FURA-2 solution. As illustrated in supplemental figure 2A for Ca^{2+} concentrations of 0 and 39 μM, we observed that the fluorescence of FURA-2 was altered with surfactant (n=5-6, supplemental figure 2B) and we applied a correction to the calibration to account for the signals in the RBL cells in the presence of surfactant. Figure 7A illustrates mast cell responses upon addition of serum and Ova. There were no differences in the baseline intracellular calcium concentrations with and without surfactant (n=5-6, figure 7B). Neither the peak nor the plateau response in calcium were significantly affected by the presence of surfactant (n=5-6, figure 7 C, D).

Figure 1: R_N, G, and H over the 20 minute period following aerosol challenge with PBS or Ova and normalized constant phase parameters over time in Ova-challenged animals. (A) R_N and (B) G are significantly different following Ova challenge than their respective controls (n= 6-8/group) at 3 minutes while (B, C) G and H are markedly higher than controls at 20 minutes. (D) Illustration of normalized parameters for the Ova-challenged animals (*p<0.05, **p<0.01).

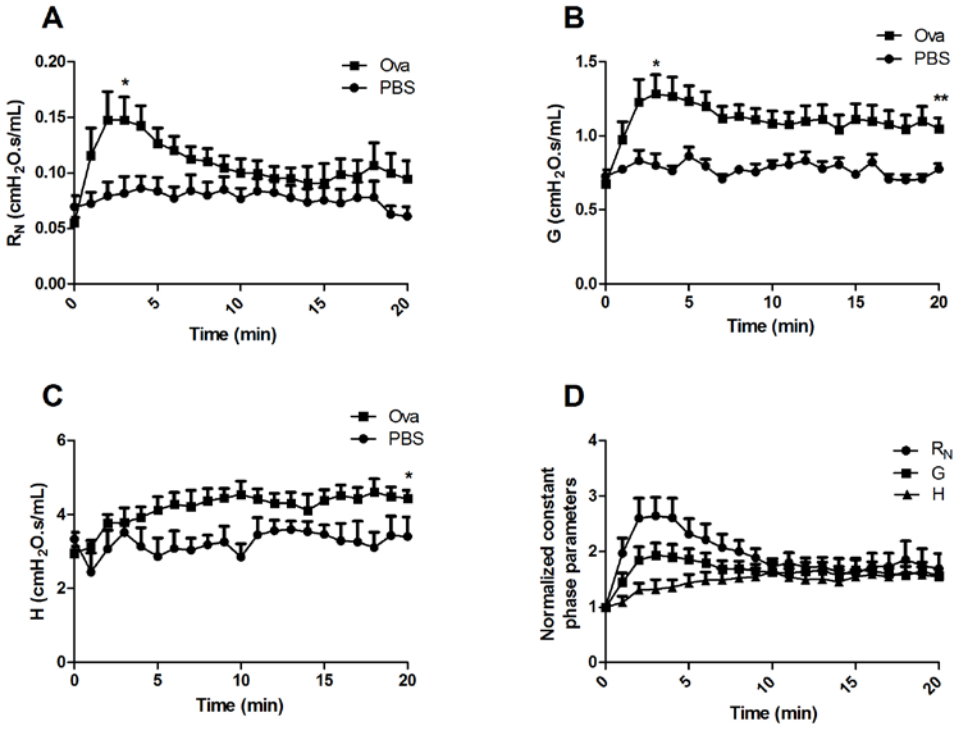


Figure 2: R_N/G, R_N/H and G/H over the 20 minute period following aerosol challenge with PBS or Ova.

While there were no differences in (A) R_N/G, (B) R_N/H and (C) G/H were significantly elevated at 3 minutes after Ova challenge (n=6-8/group). At 20 minutes there were no significant changes (*$p<0.05$).

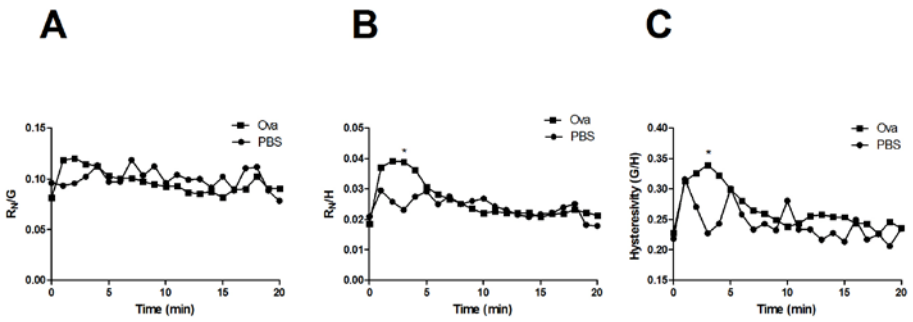


Figure 3: Airway Narrowing Morphometry at 3 and 20 minutes after aerosol challenge with PBS or Ova. Statistics were performed using the median airway size to assign airways ≥0.89 mm as medium/large airways and <0.89 mm as small airways.

(A) At 3 minutes, only larger airways in Ova compared to PBS were significantly different (C) 0.86 P_{BM} was the minimum cut-off to observe such differences. (B) At 20 minutes, only the smaller airways indicated significant differences between the treatment groups and (D) with increasing airway size, the luminal ratio difference between the treatment groups diminished. (n=5-6, *p<0.05, **p<0.01).

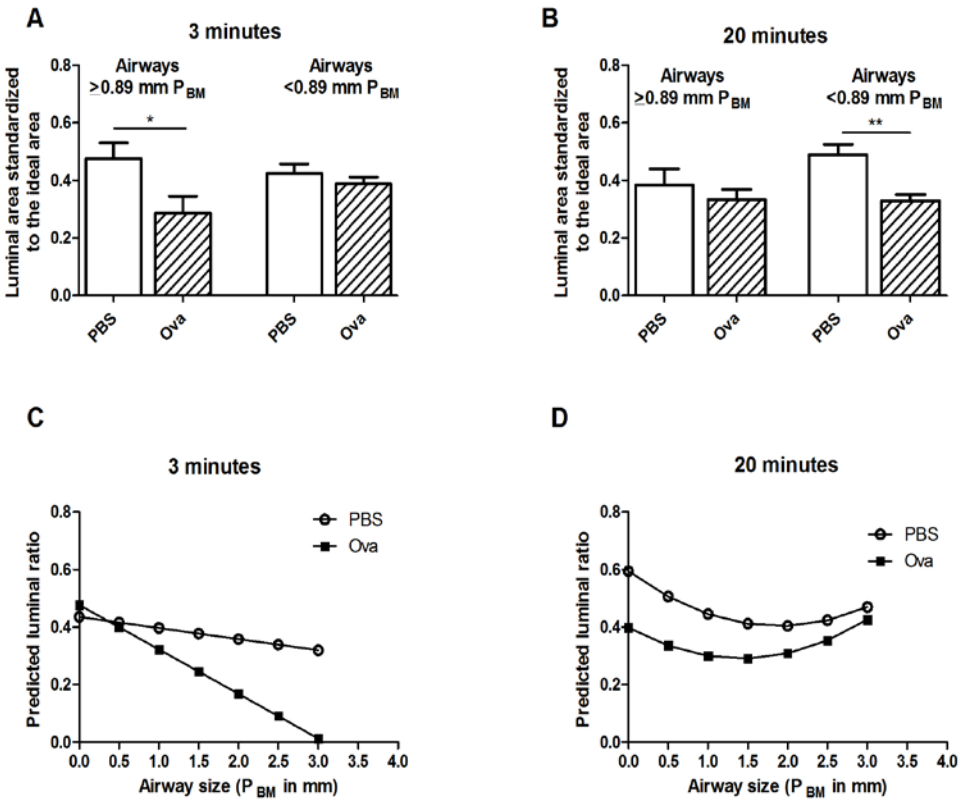


Figure 4: Airway narrowing heterogeneity at 3 min and 20 min after aerosol challenge with PBS or Ova. (A) Illustration of bronchoconstriction in PBS and Ova-challenged animal (Scaling bar = 100 µm). (B) While there were no differences to report at 3 minutes (n=5-6), (C) airway narrowing heterogeneity was greater in the Ova-challenged animals compared to controls at 20 minutes (n=4-6, *p<0.05).

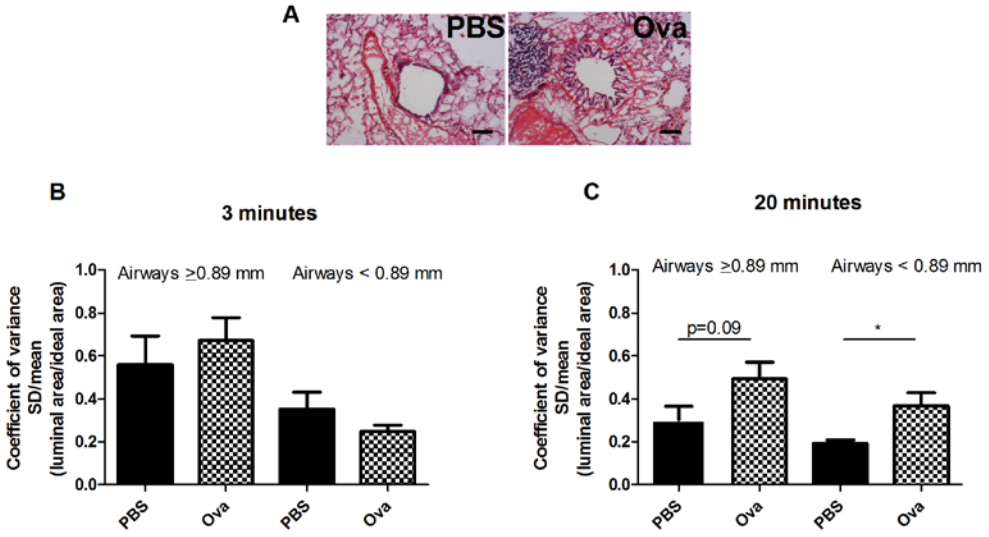


Figure 5: Surfactant effects on the constant phase parameters of AHR and corresponding morphometric assessments. The following groups were compared: PBS (n=6), Ova (n=6), Surf-PBS (n=3), and Surf-Ova (n=8). (A) R_N at 3 minutes was inhibited by exogenous, natural surfactant administration, (B) G at 3 minutes, (C) H at 3 minutes, (D) The larger airways are bronchoconstricted as assessed by morphometry but the surfactant effects did not reach significance (n=4-7, *p<0.05).

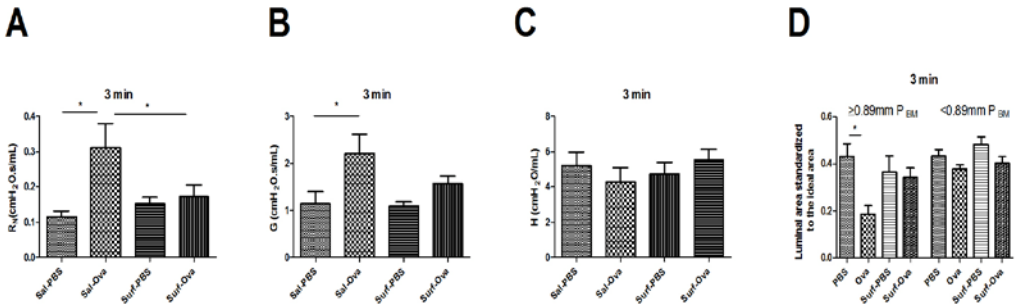


Figure 6: Mast cell–derived mediators in BAL fluid and *in vivo* assessment of responses with serotonin-mediated bronchoconstriction in the presence of surfactant

BAL was performed at 3 minutes after PBS or Ova challenge and mediators were measured by EIA or ELISA kits. (A) Cysteinyl leukotrienes (n=5-8/group) and (B) amphiregulin levels (n=7-9/group) (*p<0.05), (C) Serotonin-induced bronchoconstriction is inhibited by pre-treatment with surfactant, as observed in R_N at the peak response as well as at 5, 10, and 15 minutes after allergen challenge (n=7, *p<0.05, **p<0.01). (D) At 5 minutes into the response, there is a trend for the inhibition of the G parameter by surfactant (p=0.08) and is borderline significant at 10 minutes (p=0.065).

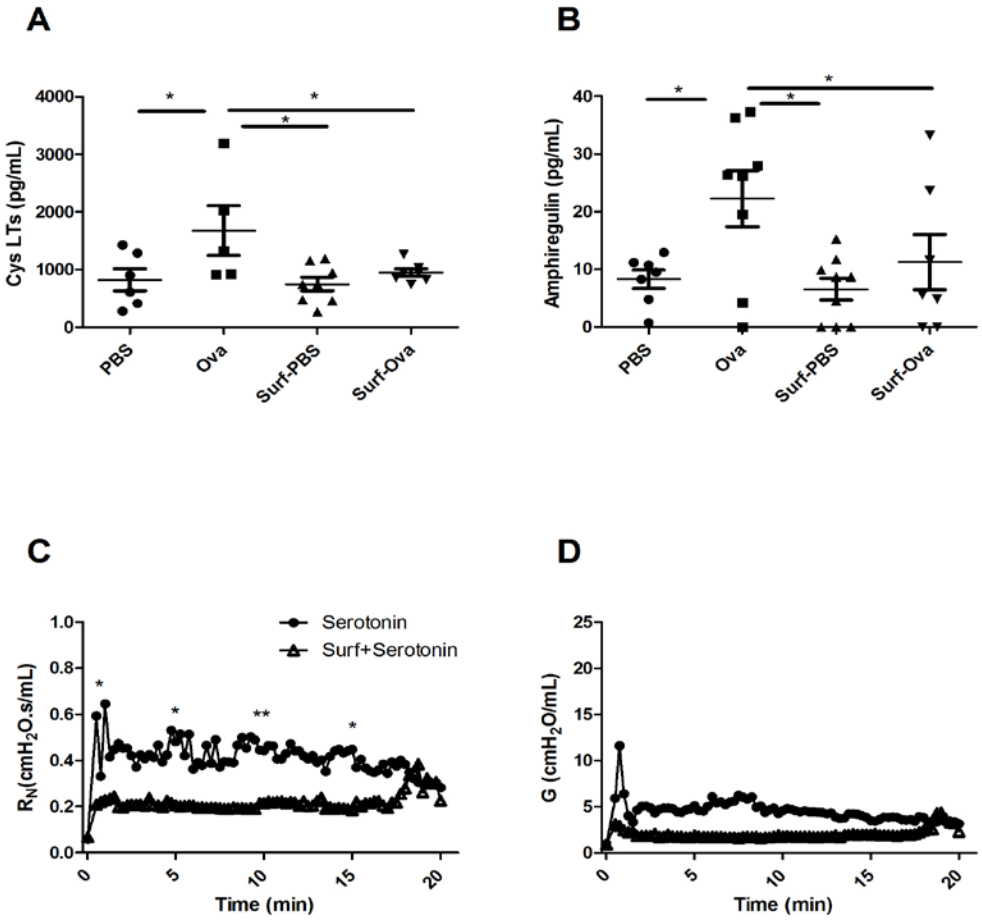


Figure 7: Effects of surfactant on RBL cell activation by Ova

(A) Images demonstrating Ca^{2+} release in mast cells, upon addition of serum and Ova. (B) There were no differences in the baseline values of the calcium with and without surfactant (n=5-6). (C) The peak increase in calcium (n=5-6) and the (D) subsequent plateau were not significantly attenuated by prior treatment with surfactant (n=5-6).

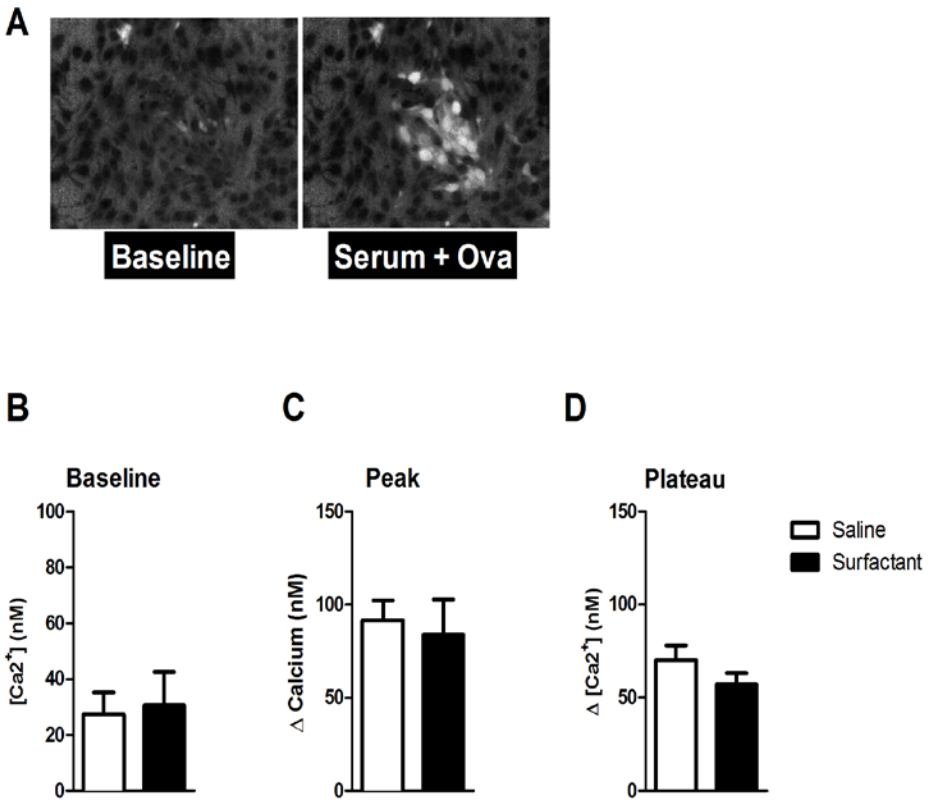
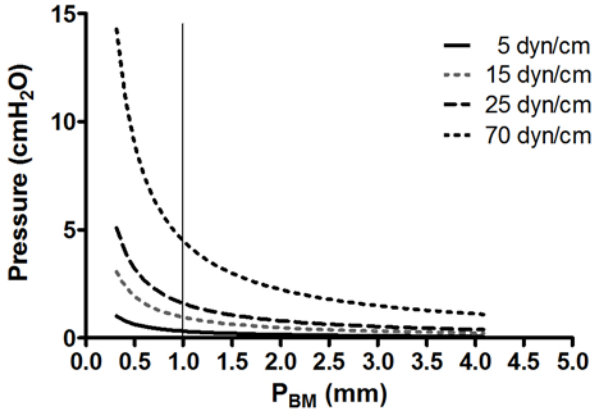
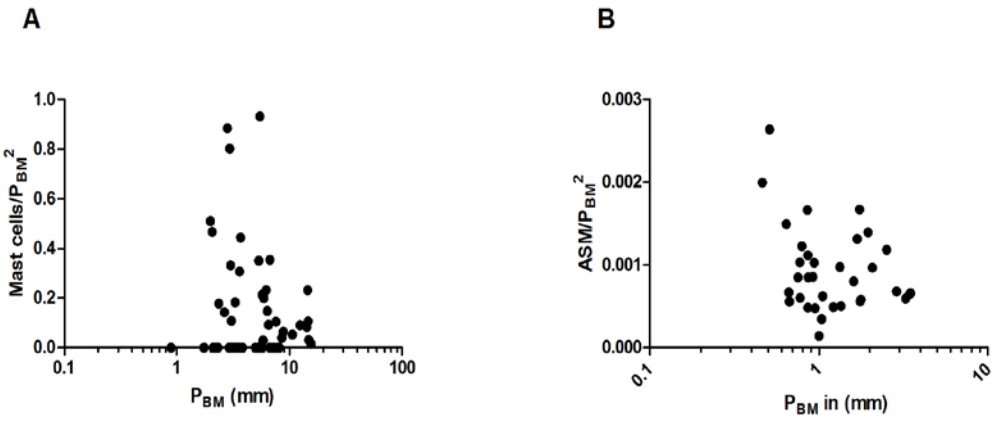


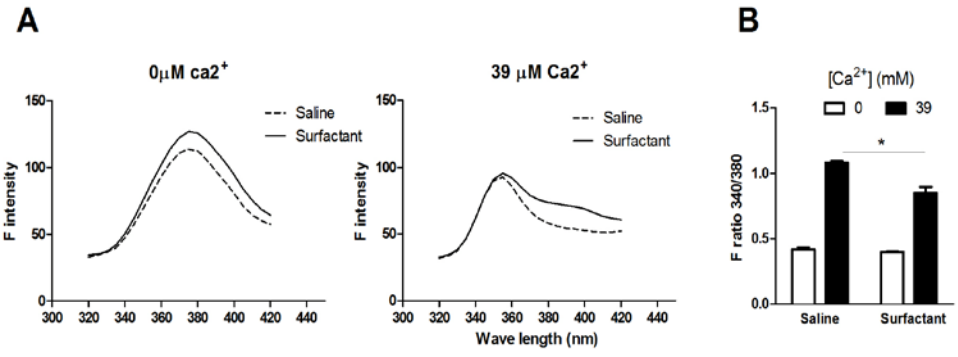
Figure 8: Possible determinants of early airway response; surface tension. We calculated the transmural pressure that might be required to overcome surface tension as a function of airway size using the Laplace relation: $P = 0.0102 \cdot \gamma / r$ where P is transmural pressure in cmH_20, γ is surface tension (ST) in dynes/cm, r is airway radius in mm and the factor of 0.0102 converts from Pa to cmH_20. The ST of water is 70 dynes/cm while the ST of fluid in the lining of the lung is between 5-30 dynes/cm.



Supplemental figure 1: Possible structural determinants of the site of the early airway response; mast cells, ASM in Ova-sensitized but unchallenged animals. (A) There was no relationship between airway size and the density of mast cells (n=8). (B) The size corrected area of ASM was not different in larger ($\geq$ 0.89 mm P_{BM}) compared to smaller airways (<0.89 mm P_{BM}) (n=6).



Supplemental Figure 2: Effects of surfactant on RBL cell activation by Ova (A) Examples of responses at 0 and 39 μM, respectively, in a cell-free mix. (B) The fluorescence of FURA-2 was altered with surfactant (n=5-6, *p<0.05).



2.8 **Discussion**

Ova exposure triggered a significant EAR in sensitized animals as determined from changes in respiratory mechanical properties of the respiratory system. The peak of the EAR occurred at ~ 3 minutes after the challenge at which time both of the constant phase parameters R_N and G were elevated. By 20 minutes, R_N was no longer significantly elevated but G and H were. Narrowing of the large airways ($\geq$ median size) assessed by morphometry was present at 3 minutes after challenge and had resolved by 20 minutes whereas the narrowing of the smaller airways (< median size) was slower in its onset. Surfactant administered prior to allergen challenge inhibited the changes in mechanical parameters reflecting large airway constriction at 3 minutes after challenge and at all subsequent time points up to 20 minutes. Surfactant inhibited the release of the mast cell-derived inflammatory mediators, cysteinyl leukotrienes and amphiregulin, at the EAR peak. *Ex vivo*, surfactant failed to inhibit Ova-induced calcium release from RBL cells, indicating that the amelioration of airway narrowing by surfactant is more likely attributable to indirect effects on mast cells.

Following Ova challenge, R_N and G rose quickly and synchronously whereas the changes in H lagged in time. The changes in R_N were transient whereas changes in G persisted over the 20 minute period of observation. R_N/G was not significantly elevated at peak EAR suggesting that these parameters may share common determinants, namely narrowing of the airways larger than the median value or that they may reflect simultaneous effects on different regions within the airway tree and lung parenchyma. The change in R_N is usually attributed to airway resistance and G to tissue damping (1;21). The elevation in G at a time when R_N had recovered is consistent with the idea that the factors contributing to R_N and G are not identical. In contrast, R_N/H and G/H were significantly different at peak EAR, suggesting that H is determined by changes in the peripheral lung tissues and beyond the airways captured by changes in R_N and G.

In order to assess the contributions of the narrowing of airways of different sizes to the parameters of the constant phase model we snap-froze lungs

following allergen challenge at a transpulmonary pressure that fixed lung volume in the tidal breathing range, using previously published methodology (20). We divided airways into two categories, larger and smaller than the median value (0.89 mm internal perimeter) in order to relate morphometric measurements of airway narrowing to the constant phase parameters. The morphometric data showed narrowing of the larger airways at the peak EAR and recovery by 20 minutes. The converse was observed for smaller airways that were narrowed significantly at 20 minutes but not at 3 minutes after challenge. These data are in concordance with the interpretation offered for the parameters of the constant phase model, although it does appear that the G parameter is sensitive to both large and small airway narrowing.

The heterogeneity of airway narrowing seems likely to be dependent on the fact that airway narrowing was induced by aerosol challenge (22) although a substantial contribution from local mechanisms and not solely non-uniform agonist delivery has also been suggested (23). There were no differences in heterogeneity of airway narrowing at 3 minutes after challenge amongst any of the groups. By 20 minutes after Ova challenge, the airway narrowing heterogeneity markedly increased in the smaller airways and showed a strong trend for increase in the larger airways. The coefficient of variation of the airway luminal sizes in the PBS-challenged group was higher at 3 minutes than at 20 minutes suggesting that there was a response in the larger airways to PBS, albeit resulting in a much smaller observable effect on respiratory system mechanics. The heterogeneity of constricted airways may be analyzed via direct visualization by morphometric analysis on frozen lungs (24), by hyperpolarized 3-helium dynamic MRI (25), high-resolution computerized tomography (26-28), mechanical measurements such as the alveolar capsule technique (29) and by forced oscillation and model fitting to whole lung impedance data (30;31). The heterogeneity of small airway narrowing observed for Ova-challenged animals correlated with the G and H parameters.

We explored several of the important potential determinants of the site and magnitude of Ova- induced airway narrowing such as mast cell density and

ASM mass. We found no clear differences in mast cell distribution across airways of increasing size and no significant airway size dependence of SM mass. There are several factors which should favor a greater narrowing of the smaller airways for an equivalent allergen challenge. Firstly, the decrease in airway radius should reduce the wall tension required to create a given transmural pressure, based on the Laplace relationship (32). For similar reasons, surface tension within the small airways should favour airway narrowing ((33) and figure 8) and the effect would be expected to be markedly enhanced by the disruption of surfactant properties by the microvascular leak of protein that has been shown to occur during the early allergic response (9). We speculate that the more sustained abnormality in peripheral lung mechanics is attributable to airway closure, favoured by high surface tension and the development of fluid menisci within some of the small airways.

The surface tension of the fluid in the lungs *in vivo* has been estimated to be between 5 and 30 dynes/cm or mN/m (34). Forty-eight hours after antigen challenge, there is reportedly a dysfunction in pulmonary surfactant (35). It was later reported that in asthmatic humans, phospholipid alteration in surfactant, particularly in phosphatidylglycerol, occurs after antigen challenge (12). Furthermore, in stable asthmatic individuals, dipalmitoyl phosphatidylcholine, the primary phospholipid found in surfactant is reduced in sputum but not BAL surfactant (36). Small airways are most at risk of closure and an intervention to decrease surface tension may decrease the risk of airway closure. In small animals the distribution of the surfactant-producing Clara cells throughout the airways suggests that surfactant may have such a role even in larger sized airways in small animals (37). In order to explore the plausibility of disruption of surfactant function within the airways by protein leakage we calculated the transmural pressure that might be expected as a result of surface tension as a function of airway size using the Laplace relation (figure 8). Based on our experimental data, surfactant had protective effects in inhibiting airway obstruction. We had postulated this effect would be most evident in the peripheral airways as this is the site where surfactant is endogenously more

abundant and has the greatest effect on transmural pressure. However, the inhibition of the peak EAR, as well as the reduction of bronchoconstriction of the larger airways, indicated that surfactant was playing a role in larger airways.

Synthetic surfactant has been described to inhibit early allergic airway responses in human subjects, presumably related to effects on surface tension. Collectins have been associated with the inhibition of various mediators including the IgE-mediated degranulation of mast cells (38). However surfactant proteins A and D are not present in the surfactant preparation that we used. A protective role of inhaled synthetic, protein-free, phospholipid-based surfactant in the early allergen-induced response in humans has also been described (39). To address the possibility that the surfactant we employed, lacking immunomodulatory proteins, may have a role in modulating inflammatory mediators, we assessed the concentrations of CysLTs in the BAL in BN rats immediately after the EAR peak was reached. CysLTs were significantly inhibited by exogenous surfactant administration. We observed similar results with amphiregulin. Amphiregulin, an epidermal growth factor receptor ligand is reported to be present in 70% of human asthmatic mast cells (40). It is released by epithelial and mast cells and could potentially be important in the EAR as it has been reported to be elevated after an acute asthma attack in humans (41). Interestingly, human cord blood-derived mast cells (CBMCs) secrete amphiregulin upon IgE crosslinking (40).

We examined the effects of this surfactant preparation on the activation of RBL-2H3 cells, a frequently used model for the mast cell. The effects of surfactant appear to be indirect on the mast cells since activation by Ova and sensitized serum *ex vivo* was not reduced by treatment with surfactant. However bronchoconstriction by mechanical effects *per se* does not lead to release of mast cell mediators (42) and it is therefore not clear how a reduction in surface tension *per se* may have reduced mediator release. Surfactant also reduced the serotonin-induced central airway narrowing. Together, the data suggest that collectin-free surfactant inhibit the release of mast cell mediators *in vivo*.

In summary, ovalbumin exposure induced a significant EAR, in which the peak of the response occurred as early as ~ 3 minutes after the challenge. Mechanical assessments and morphometry on frozen tissues indicated that the narrowing of the large airways occurred transiently and resolved quickly whereas the peripheral airway narrowing was more persistent. Changes in R_N appear to reflect narrowing of airways greater than the median-sized airway whereas G is affected by airway narrowing in airways that are larger or smaller than the median-sized airway. Exogenous surfactant administration decreased the peak R_N and morphometric larger airway narrowing in Ova-challenged animals, attributable most likely to indirect action on the mast cells. These results suggest that there may be a role for exogenous, collectin-free surfactant in the treatment of acute asthma in mechanically-ventilated subjects.

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Chapter 3

Sites of allergic airway smooth muscle remodeling and hyperresponsiveness are not associated in the rat

3.1 **Prologue**

The relationship between airway hyperresponsiveness and airway remodeling is not clear. With an established model of allergic "asthma" using the Brown Norway (BN) rat, and a model of airway and lung parenchymal mechanics (constant phase model), our aim was to determine the relationship between airway mechanical function, including airway hyperresponsiveness, and airway remodeling. An increase in goblet cell numbers and increased smooth muscle mass were used to assess remodeling as they are markers of remodeling and they mediate allergen-induced changes in mechanical properties. In addition we studied the effects of budesonide (Bud) on airway structure-function relationships, reasoning that this potent anti-inflammatory agent might have effects on remodeling and airway responsiveness that may be unrelated.

3.2 **Abstract**

Rationale: The cause and effect relationship between airway smooth muscle (ASM) remodeling and hyperresponsiveness (AHR) following allergen challenge is not well established.

Objectives: Using a rat model of allergen-induced ASM remodeling we explored the relationship between the site of ASM remodeling and AHR.

Methods: BN rats, sensitized and challenged (3x at 5-day intervals) with ovalbumin, were intranasally administered 0.1 mg/kg budesonide, 24 and 1h prior to challenge. Airway responses to aerosolized methacholine were assessed 48 h or 1 week after 3 challenges. Airways were stained and analyzed for the total airway wall area, the area of smooth muscle specific α-actin and goblet cell hyperplasia and the constant phase model was used to resolve the changes in respiratory system mechanics into large airway and peripheral lung responses.

Main results: After three ovalbumin challenges, there was a significant increase in ASM area and in the total wall area in all sized airways as well as an increase in goblet cells in the central airways. Budesonide inhibited ASM growth and central airway goblet cell hyperplasia following ovalbumin challenges. Budesonide also inhibited small but not large airway total wall area. AHR was attributable to excessive responses of the small airways whereas responsiveness of the large airways was unchanged. Budesonide did not inhibit AHR after repeated challenges.

Conclusions: ASM remodeling induced by repeated allergen challenges involves the entire bronchial tree whereas AHR reflects alterations in the lung periphery. Prevention of ASM remodeling by corticosteroid does not abrogate AHR.

Key words: asthma, Brown Norway rat, budesonide, constant phase model

3.3 **Introduction**

Asthma is a chronic inflammatory condition. When asthma is allergen-driven it is a Th-2 type disease and is characterized by features that include reversible airway obstruction, chronic inflammation, airway hyperresponsiveness (AHR) and airway remodeling. Airway remodeling comprises structural changes in virtually all cells of the airway wall. Prominent among these changes are epithelial proliferation (31) and denudation (21), reticular basement membrane thickening (17), goblet cell metaplasia (19), sub-epithelial fibrosis (32), increase in airway smooth muscle (ASM) mass (11), and mucus gland hyperplasia and hypertrophy (7). The mechanisms of airway remodeling are complex and likely involve lipid mediators, cytokines, chemokines and growth factors released from both inflammatory and structural cells (12).

Airway hyperresponsiveness (AHR) is a reflection of the enhanced capacity of the airways to respond to bronchoconstrictors and as such, may be predominantly a reflection of the ability of the ASM to overcome intrinsic impedances to airway narrowing (36). Although it seems intuitively obvious that an increase in ASM mass should lead to AHR, data supporting this hypothesis have been elusive. Further uncertainty about the relationship between remodeling of ASM and AHR is based on the fact that a change in phenotype with loss of contractile proteins may follow hyperplasia (20; 26). Additionally, the degree of remodeling and changes in ASM properties may not be uniform throughout the airway tree (26). Given the current view that airway remodeling, and ASM remodeling in particular, has key therapeutic importance we felt that it was important to assess whether AHR was dissociable from ASM remodeling. Alternative mechanisms for AHR have been proposed. For example, AHR may reflect geometrical effects of remodeling of tissues internal to the ASM itself (43) or through encroachment on the lumen by mucus. Sub-epithelial fibrosis has been argued to enhance airway narrowing through impeding airway folding (44). Fibrosis has also been proposed to enhance AHR by stiffening the airway and reducing the effects of the cyclical breathing movements on the stretching of ASM associated with these movements (13; 36).

In order to investigate the relationship between ASM growth and the alterations in airway responsiveness to methacholine (MCh), we used a well characterized rat model of "allergic asthma" in which reproducible remodeling of ASM occurs after repeated inhalational challenges with allergen (33; 34; 38; 46). Using morphometric techniques we identified the site of ASM remodeling induced by allergen challenges and compared it to the site of AHR to MCh. We also quantified the changes in goblet cell number, the change in airway wall area and the subepithelial airway wall area of the airway tissues inside the inner aspect of the ASM bundles because of the postulated importance of these aspects of remodeling to AHR (27). To determine the site within the respiratory tree responsible for AHR we applied the constant phase model of respiratory mechanics to partition the changes in mechanics following aerosol challenge with MCh that were attributable to either the large airways or the peripheral compartments of the lung. We also compared our findings to the more frequently used single compartment model to evaluate resistance (Rrs) and elastance (Ers) responses. As an additional test of the mechanistic link between ASM growth and AHR we administered a topical corticosteroid, budesonide, prior to allergen challenges, reasoning that the drug treatment might affect AHR and remodeling differently. AHR also follows a single allergen challenge during which time ASM growth does not have time to occur. Inflammatory mechanisms and, in particular, the cytokine interleukin-13 have been implicated in this form of AHR (39; 42). We were interested in examining whether the changes in mechanical properties induced under these circumstances were similar to those induced by repeated allergen challenges accompanied by ASM remodeling. We performed a multiplex assay of cytokines to examine the relationship between the AHR following repeated allergen challenge to potential biomarkers present in bronchoalveolar lavage (BAL) fluid.

3.4 **Methods**

Protocol for sensitization and challenge

Male Brown Norway rats (SsN substrain) (Harlan/UK) were sensitized subcutaneously with 1mg ovalbumin (Ova) and 100 mg aluminum hydroxide dissolved in 1 ml of phosphate buffered saline (PBS) (38). Concurrently, the rats were injected intraperitoneally with $2x10^9$ *Bordetella pertussis* heat-killed bacteria (provided by T. Issekutz, Dalhousie, University, Halifax, NS, Canada) (38). Aerosol challenges, with either 5% Ova or the PBS control, were performed in endotracheally intubated animals breathing spontaneously under light pentobarbital anesthesia. The endotracheal tube was placed in a small plexiglass box into which aerosol was introduced from a Hudson nebulizer with an output of 0.15 ml/min. Two protocols of allergen challenge were followed. In the first protocol, rats were challenged once and studied at 24 hrs after the challenge. In the second protocol, rats were challenged a total of 3 times with Ova, or its control (PBS), at 5-day intervals (38) and studied 48 h or 1 week after the final challenge (supplemental figure 1). The study protocols were approved by the Animal Care Committee of McGill University (Montreal, QC, Canada).

Treatment with budesonide

Fourteen days after Ova sensitization as described above, rats were administered with either 0.1 mg/kg budesonide, or its vehicle, saline, intranasally 24 h and 1 h prior to Ova challenge. Animals in an additional group were sacrificed at 1 week after 3 challenges to test whether AHR and ASM and epithelial cell remodeling persisted at this time point.

Budesonide (Bud) (0.1 mg/kg) was prepared from a suspension manufactured for inhalation (Pulmicort Nebuamp, Astra Zeneca, Mississauga, Ontario, Canada) and was administered intranasally in 100 μL of saline.

Assessment of the lung deposition of an intranasally administered suspension

Evans blue dye (500 μg) was delivered intranasally in the same manner as budesonide. The animals were lightly anesthetized with isofluorane during the delivery. The animals were sacrificed 1 h later with anesthetic overdose. The trachea and lungs were removed and Evans blue dye was extracted with formamide in a 60^{o}C water bath for 24 h. Optical density was assessed at 620 nm with a spectrophotometer. Extracted Evans blue was quantified on a standard curve. We determined that 59.5% of the administered dose was deposited in the lungs (supplemental figure 2).

Airway responses to methacholine challenge

Airway responses to methacholine (MCh) (Sigma Chemicals, St Louis, MO), were assessed using the linear single compartment and constant phase models using the Flexivent system (Scireq, Montréal, QC) at 48 h or 1 week after 3 Ova challenges, or 24 h after a single challenge. Animals were anesthetized with xylazine (10 mg/kg) and pentobarbital (35 mg/kg) and paralysis was induced with 1mg/kg pancuronium bromide intraperitoneally (Sandoz Canada Inc, QC). The animals were placed on a heating pad and their temperature was monitored to ensure that it remained between 34-36^{o}C. A PEEP (positive end-expiratory pressure) was maintained between 2 and 2.5 cmH_2O and the animals were ventilated at a tidal volume of 8mL/kg and a breathing frequency of 90 breaths/minute. The linear single compartment model was used to assess total respiratory system resistance and elastance whereas the constant phase model was used to further partition responses within the lungs. The latter model fits the data to an equation that has four parameters and these are estimated by solving the following (15):

$$Zrs(f)=R_N + j\times 2\pi f\times Iaw + (G-j\times H)/(2\pi f)^{\alpha}$$

where Z is input impedance and expresses the combined effects of resistance, compliance and inertance as a function of frequency, R_N is Newtonian “airway”

resistance, Iaw is airway inertance and is dominated by the mass of gas in the central airways, and impedance of tissues is accounted for by both G and H. G (tissue damping) is closely related to peripheral airway and tissue resistance and reflects energy dissipation in the lung tissues, j is an imaginary number, H is tissue elastance and reflects energy storage in the tissues, α is $2/\pi \tan^{-1}$ (H/G) and f is respiratory frequency (15).

Bronchoalveolar lavage fluid and assessment of inflammation

Bronchoalveolar lavage (BAL) (5 x 5mL aliquots of PBS) was performed following the assessment of airway responses. Cells were pelleted by centrifugation from the 5 aliquots and re-suspended in PBS for cell counts. Cytospins were prepared using a cytocentrifuge (Shandon Cytospin® 4 cytocentrifuge, Thermo Scientific, Waltham, MA) and were stained with Diff-Quik® Stain Set (Dade Behring, Newark, DE) to assess inflammation.

Cytokine Analysis

To assess the relationship of AHR and remodeling to inflammatory mediators we performed a multiplex cytokine analysis on the first 5 mL of BAL fluid samples retrieved at the 48 h time point in the following groups: PBS, Ova, and budesonide + Ova, using a multiplex rat cytokine kit (LINCOPLex, LINCO Research/Millipore, St Charles, MO) as performed previously (5) and after concentrating the samples 20-fold. The following cytokines/chemokines were measured: eotaxin, RANTES, GRO KC (growth-related oncogene KC), IL-1α, IL-1β, IL-13, IL-4, IL-5, IL-6, IL-9, MIP-1 α (macrophage-inflammatory protein 1 α).

Histology

For the assessment of remodeling the left lungs were fixed overnight in formalin at 25 cmH_2O and were paraffin-embedded. Histological slides were prepared from 5 μm mid-sagittal and para-hilar sections to sample medium and small

airways and large airways, respectively. The tissue sections were stained for smooth muscle-specific α -actin (α -SMA) to assess the increase in ASM mass. The mass of ASM was estimated from the area of smooth muscle-specific α -actin immunoreactivity as previously described (30; 38). Isotype controls were performed and showed no staining, confirming the specificity of smooth muscle-specific α –actin staining. Airways ≥1mm basement membrane perimeter (P_{BM}) were considered to be medium and/or large airways and <1mm P_{BM} as small airways (30). In brief, sections were stained with a mouse monoclonal antibody to α-SMA (clone 1A4; Sigma-Aldrich Co.) as well as a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). The signal was developed with Vector® Red. The area of ASM was traced using a camera lucida side arm microscope attachment and was digitized with commercial software (SigmaScan Pro 5, Ashburn, VA). The area of ASM was normalized for airway size by dividing by the square of the P_{BM}.

To assess goblet cell hyperplasia, sections were stained with Periodic-Acid- Schiff (PAS) (Sigma Chemicals, St Louis, MO). The quantitative assessment was performed by counting the number of PAS positively-stained cells and normalizing by the basement membrane perimeter.

The total airway wall area was assessed by measuring the area of the airway from the luminal surface of the epithelium to the adventitial surface and correcting for the square of the basement membrane perimeter. The area of the airway wall inside the ASM layer was determined by subtracting the luminal area at the tip of the epithelium from the area at the inner aspect of the ASM and correcting for the square of the basement membrane perimeter.

Statistical analysis

Data are presented as mean ± SEM. To ensure adequate morphometric estimates, we assessed the number of airways required for a stable estimate of the means for α -SMA normalized area and goblet cells for each animal (supplemental figure 4). The statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA). For the ASM, goblet cell, total

airway wall area, and airway wall area inside the smooth muscle bundles analyses, one-way ANOVA was performed followed by a Newman-Keuls test. For the physiological measurements, a repeated measures ANOVA was performed followed by a Bonferroni correction. A p-value of <0.05 was considered significant.

A linear discriminant analysis was performed for the cytokines/chemokines assessed in the BAL fluid with the multiplex kit and statistical analysis was performed using R (R Foundation for Statistical Computing, Vienna, Austria). The linear discriminant analysis was performed to determine whether the cytokine results in the various treatment groups provided sufficient information to discriminate or classify treatment groups as separate from one another (supplemental figure 3). The first linear discriminant function discriminated the Ova from the PBS and Bud+Ova groups. The second linear discriminant function discriminated the PBS from the Bud+Ova group.

3.5 **Results**

Allergen-induced changes in airway responsiveness to methacholine; single compartment model

At 24 h after a single Ova challenge, AHR was detectable as exaggerated responses in Ers, to MCh challenge, (Figure 1A). There were no significant changes in Rrs observed (Figure 1B). At 48 h after three Ova challenges, the animals had a higher Rrs to aerosolized MCh (Figure 1D) and a significantly higher Ers than did the PBS-challenged animals (Figure 1C). The increase in responsiveness to MCh was no longer present at one week after the three Ova challenges (data not shown). AHR after a single Ova challenge was inhibited by budesonide administered prior to Ova challenge ($p<0.05$, Figure 1A).

Allergen-induced changes in airway hyperresponsiveness; constant phase model

To resolve the site of airway responsiveness to the large conducting airways or the more peripheral lung, we analyzed responses to MCh using the constant phase model. Ova challenge did not alter the responses in R_N following MCh challenge. Neither a single Ova challenge (Figure 2A) nor repeated Ova challenges augmented the response in R_N when compared to PBS controls (Figure 2B and data not shown for the 1 week time point).

At 24 h after a single challenge, there were no significant differences in G amongst the three study groups (Figure 3A). However, at 48 h after three Ova challenges, G was higher than in the PBS group at the highest 2 doses of methacholine ($p<0.05$ in Figure 3B). This increase was not prevented by budesonide (Figure 3B). At 1 week, the Ova-challenged animals no longer had a significantly elevated G compared to the controls (data not shown).

At the 24 h time point after a single challenge, the Ova-challenged group had an increased H compared to the PBS group in response to MCh ($p<0.05$, figure 4A) and this change was not inhibited by budesonide (Figure 4A). The H parameter in the Ova-challenged animals was not elevated at either 48 h (Figure 4B) or 1 week after three Ova challenges (data not shown).

Bronchoalveolar lavage fluid inflammatory cells

At 24 h after a single challenge, the Ova-challenged animals had significantly higher eosinophil numbers than the PBS-challenged animals which was not reduced by budesonide ($p<0.05$, Figure 5A). At 48 h after three Ova challenges eosinophilia was present and was not reduced by budesonide (Figure 5B). While the total numbers of cells in the various treatment groups were reduced at 1 week compared to the 48 h time point, the total number of cells and macrophages in the Ova-challenged animals were still significantly higher when compared to the PBS challenged animals ($p<0.05$, Figure 5C).

Airway Smooth Muscle Growth

We determined the extent and site of remodeling of ASM after three Ova challenges at 48 h and 1 week (Figure 6B). At 48 h, the ASM area in the Ova animals was significantly greater than in PBS animals in both the central airways ($p<0.05$) and peripheral airways ($p<0.05$). Budesonide inhibited the increase in ASM area induced by Ova-challenge in both central and peripheral airways ($p<0.05$, Figure 6B**).** The increased ASM was maintained for at least 1 week in the peripheral airways ($p<0.05$) and showed a trend for persistence in the central airways ($p=0.07$).

Total Airway Wall Area and Subepithelial Airway Wall Area

In the central airways, the total airway wall area was significantly increased in the repeated OVA-challenged animals compared to the controls ($p<0.05$, Figure 7A). In the peripheral airways, a similar pattern was observed ($p<0.01$, Figure 7A). Budesonide inhibited the increased total airway wall area in the peripheral

airways ($p<0.05$) but did not reduce central total airway wall area (Figure 7A). There were no significant observable differences in subepithelial airway wall area inside the ASM bundles after repeated Ova challenges (Figure 7B).

Goblet cell hyperplasia

The Ova animals had an increased number of goblet cells/P_{BM} in the central airways compared to the PBS-challenged controls which was attenuated by budesonide (Figure 8B, $p<0.05$). The goblet cell numbers were persistently elevated in the central airways by one week after three Ova challenges but increased even further in the peripheral airways when compared to 48 h after repeated allergen challenges ($p<0.0001$, Figure 8B).

Cytokine/Chemokine Analysis

Given the apparently stronger relationship between inflammation and AHR in comparison to remodeling and AHR, we performed a linear discriminant analysis on mediators in BAL samples collected 48 h after 3 challenges. From the plot of the first 2 discriminant functions, we found that the first function clearly discriminated between the PBS-challenged group and budesonide + Ova groups versus Ova (Supplemental figure 2). Meanwhile, the second function discriminated between budesonide + Ova and the PBS group (Supplemental figure 2). The cytokines that were dominant in the discrimination of the Ova group were IL-1β, IL-13, IL-4, and RANTES, as determined by the first function, whereas the cytokines that were dominant in discriminating the budesonide + Ova group from the PBS group comprised IL-9, eotaxin, IL-6, and IL-4.

Fig 1: (A) Total respiratory system resistance elastance (Ers) and (B) resistance at 24 h after a single challenge (n=9), (C) Ers and (D) Rrs at 48 h after multiple challenges (n=6-10). Repeated measures ANOVA was performed followed by Bonferroni correction. Data expressed as mean +/- SEM. *p<0.05, #p<0.05 comparison between Bud+Ova with Ova at the 24 h.

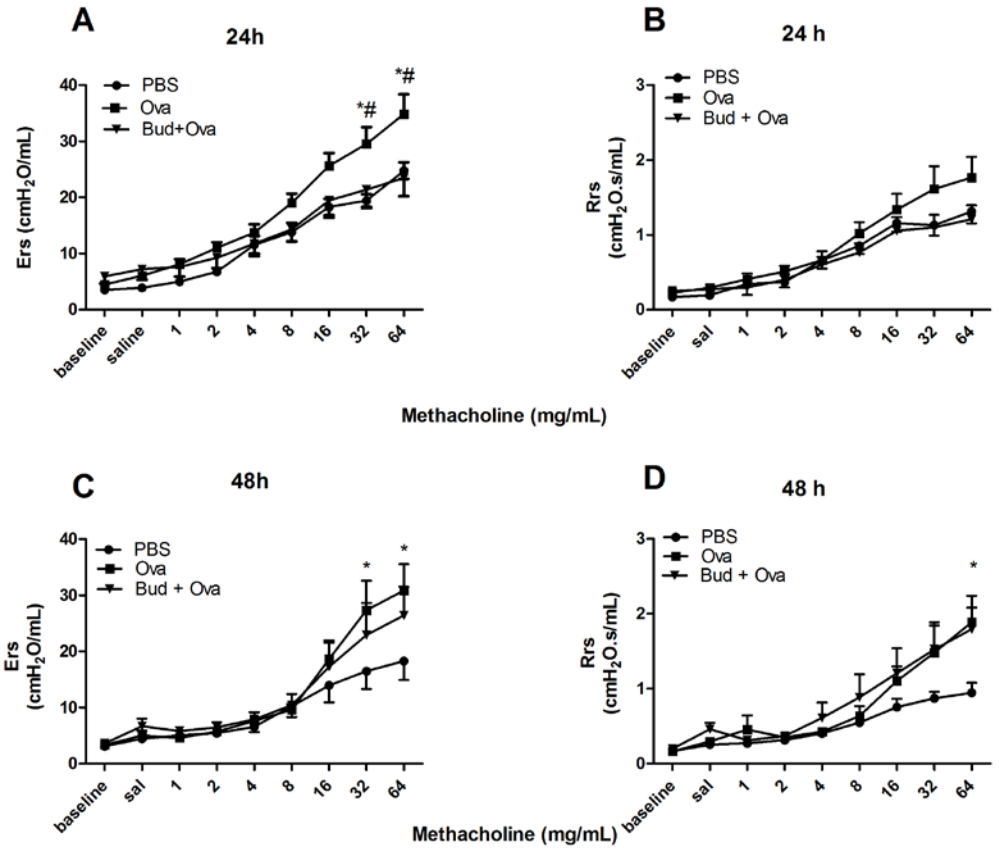


Fig 2: Constant phase model parameter, Newtonian resistance (R_N), at (A) 24 h after a single challenge (n=9-10) and (B) 48 h after multiple challenges (n=7-9). Repeated measures ANOVA was performed followed by Bonferroni correction. Data expressed as mean +/- SEM.

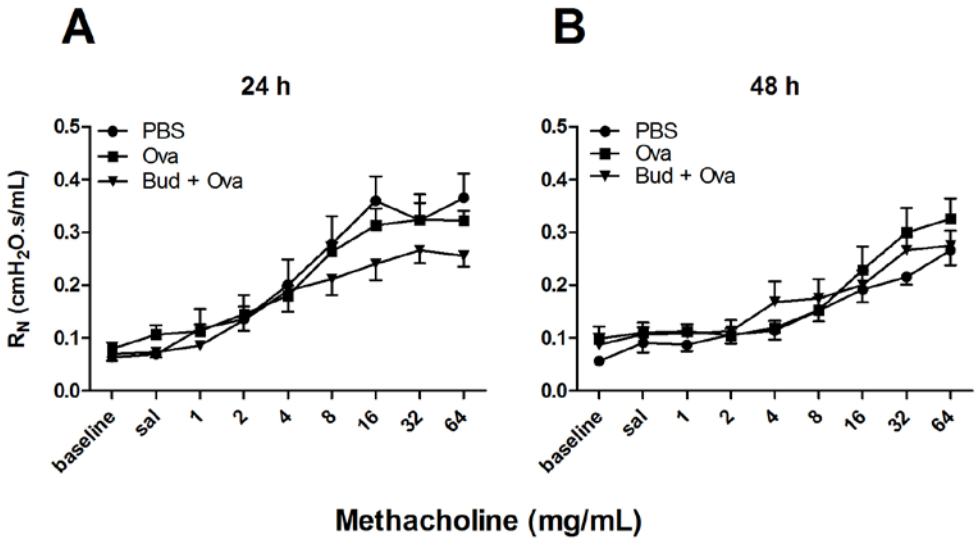


Fig 3: Constant phase model parameter, tissue damping (G) at 24 h after a single challenge (A) and 48 h after multiple challenges (B). Repeated measures ANOVA was performed followed by Bonferroni correction. Data expressed as mean +/- SEM, *p<0.05.

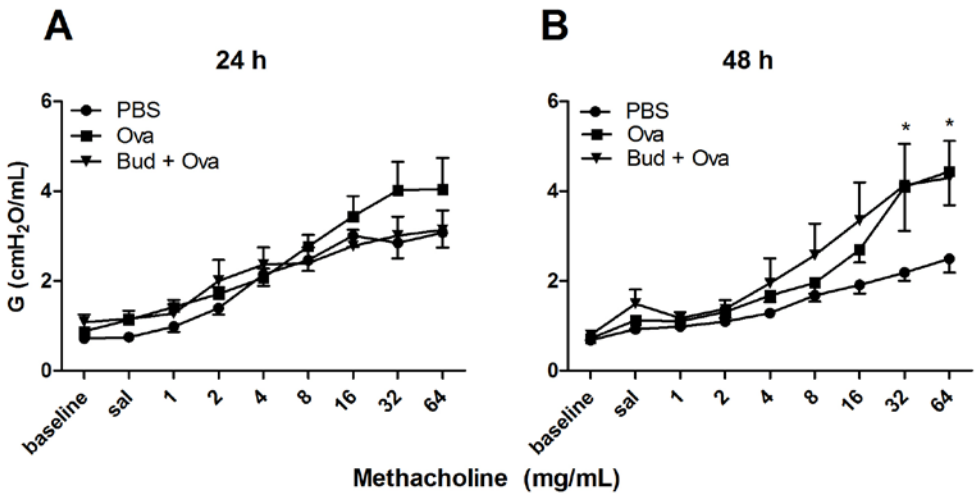


Fig 4 : Constant phase model parameter, tissue elastance (H), at 24 h after a single challenge (A) and 48 h after multiple challenges (B). Data expressed as mean +/- SEM.

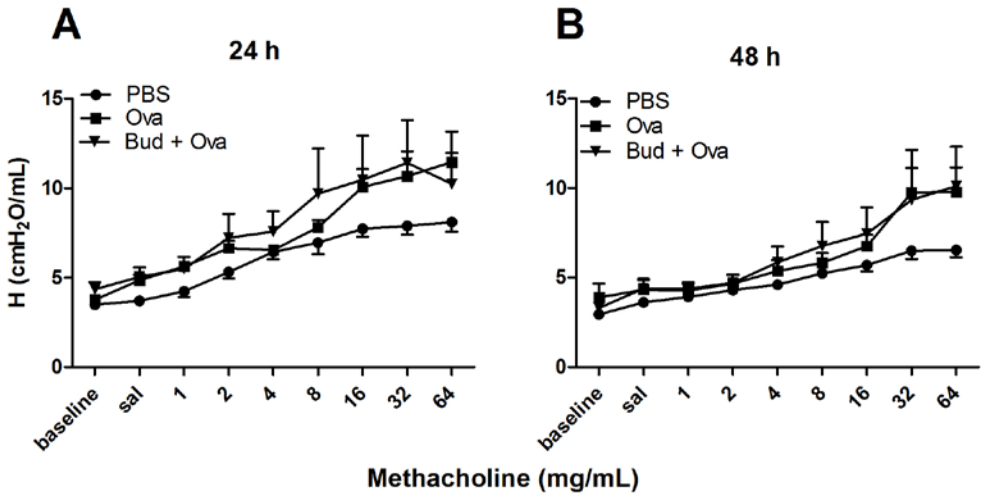


Fig 5: (A) Differential cell count from the bronchoalveolar lavage at 24 h after a single challenge (n=9-11), (B) 48 h after multiple challenges (n=5-13), (C) 1 week after multiple allergen challenges. Data expressed as mean +/- SEM, *p<0.05.

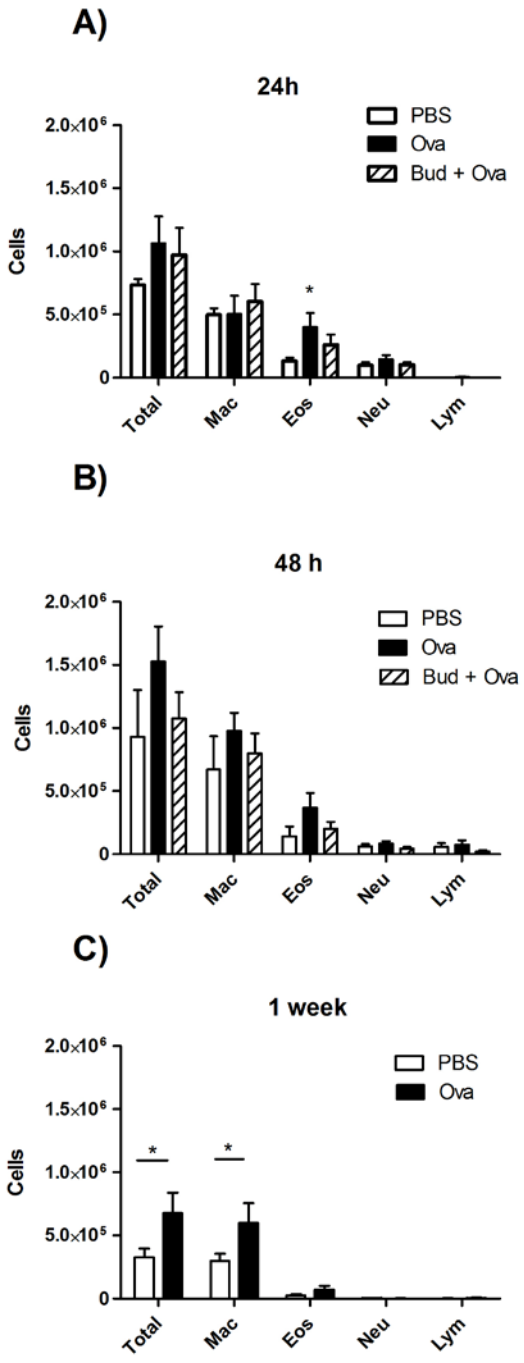


Fig 6 (A) Illustrative images (100x magnification) of the treatments: PBS, Ova, Ova at 48 h. The scaling bar is 200μm in length. (B, left): Increase in smooth muscle mass after multiple Ova challenges at 48 h in the central airways (n=8, One way ANOVA, Newman-Keuls post-hoc,* $p<0.05$). (B, right): Increase in smooth muscle mass after multiple Ova challenges at 48 h and 1 week in the peripheral airways (n=8, One way ANOVA, Newman-Keuls post-hoc, * $p<0.05$) Data expressed as airway smooth muscle mass area normalized for basement membrane perimeter squared (mean) + SEM.

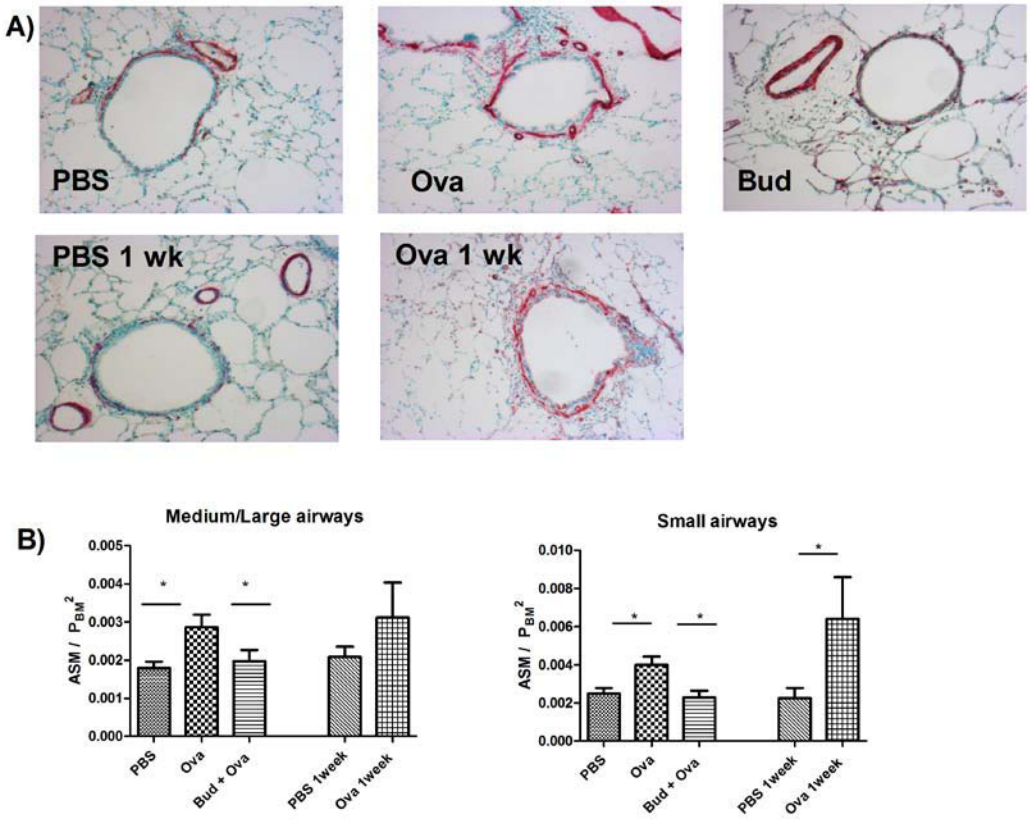


Figure 7: (A) Total airway wall area (outer airway area – luminal area)/P_{BM}^2 for the treatment groups: PBS, Ova, Bud + Ova (n=6-8, One way ANOVA, Newman-Keuls post-hoc, *p<0.05). (B) Subepithelial airway wall area ((area at the inside of ASM bundles-luminal area)/ P_{BM}^2) (n=6-8, One way ANOVA, Newman-Keuls post-hoc, *p<0.05, **p<0.01).

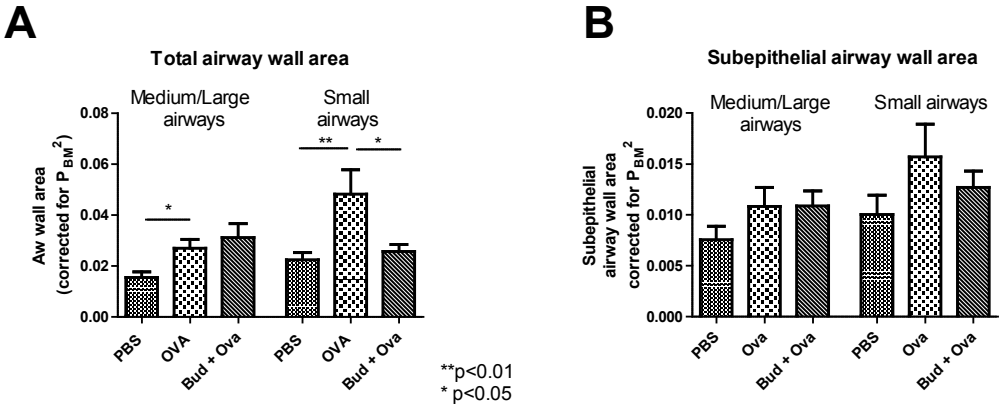
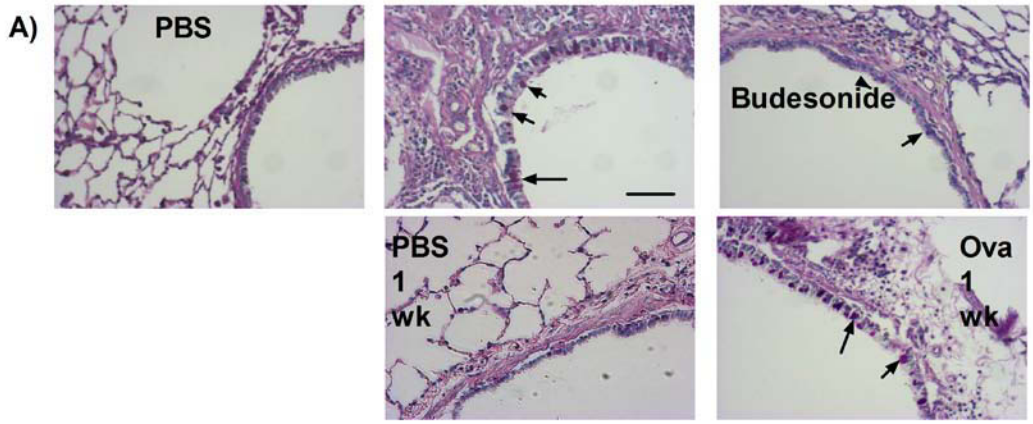
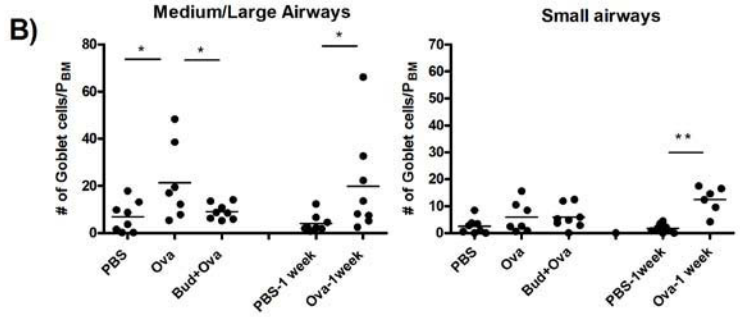


Fig 8 (A) Illustrative image of Periodic acid Schiff staining (200x magnification). Arrows are pointing towards stained goblet cells. The scaling bar is 100µm in length.

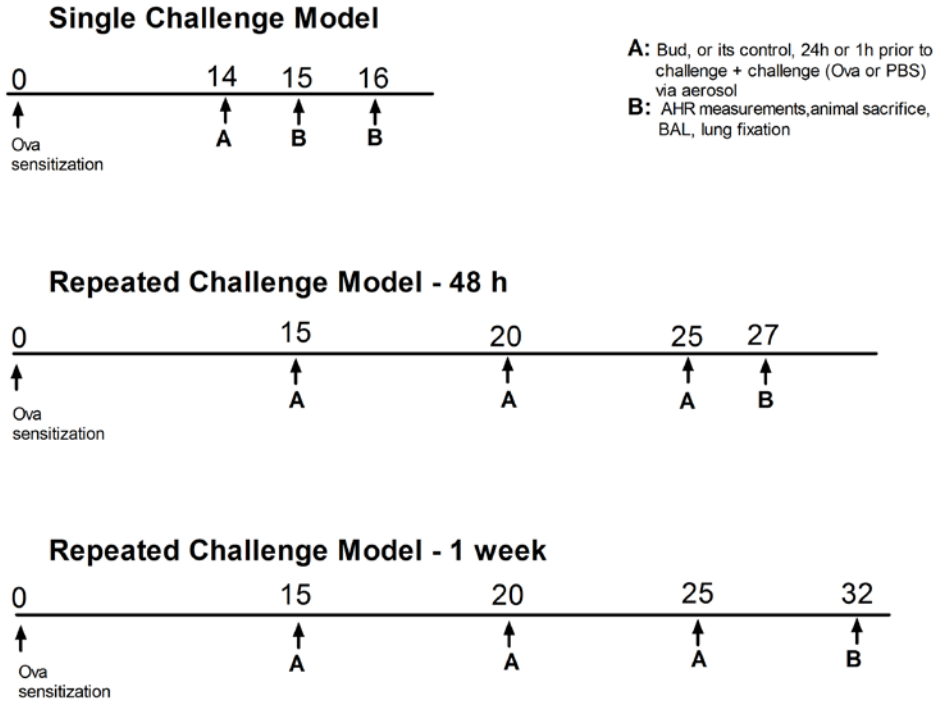
(B, left): An increase in the number of goblet cells after multiple Ova challenges at 48 h and 1 week in the central airways (n=8, One way ANOVA, Newman-Keuls post-hoc,* $p<0.05$). Data expressed as log-transformed goblet cells per basement membrane perimeter + SEM.

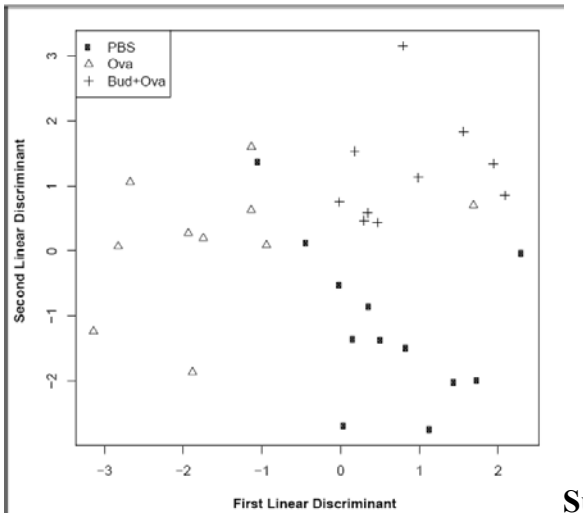
(B, right): Number of goblet cells after multiple Ova challenges at 48 h and 1 week in the peripheral airways (n=8, One way ANOVA, Newman-Keuls post-hoc,*$p<0.05$, ***$p<0.0001$). Data expressed as log-transformed goblet cells per basement membrane perimeter + SEM.





Supplementary figure 1.

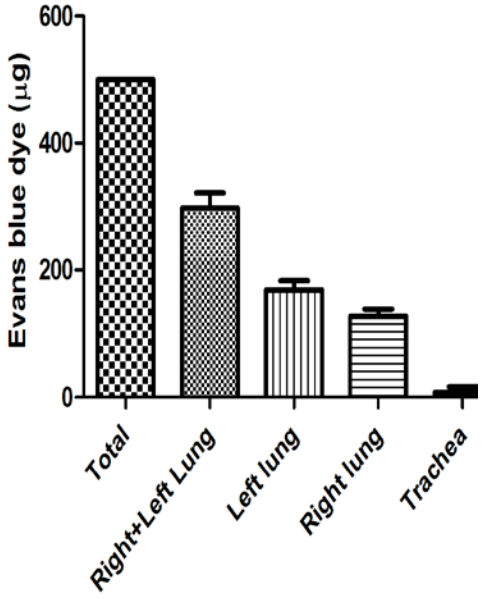


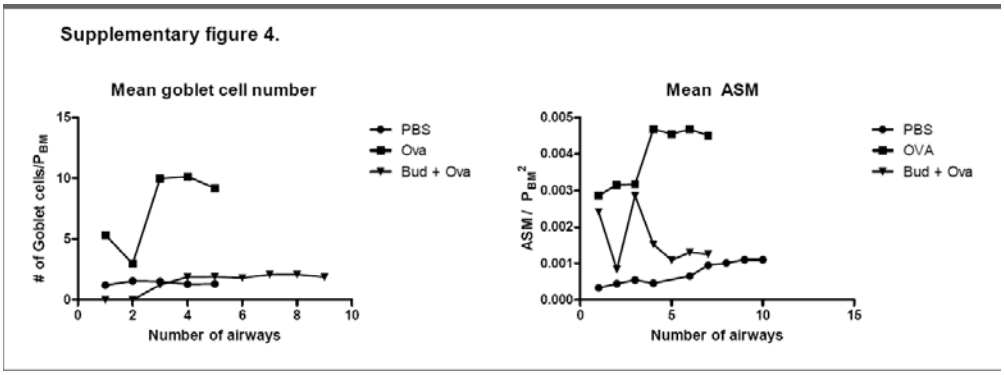


Supplementary figure 3:

Discriminant Function Scores: Classification of treatment groups in different quadrants according to the levels of cytokines expressed in the BAL.

Supplementary fig 2: Evans Blue Dye





3.6 **Discussion**

Repeated allergen challenges in the sensitized BN rat caused airway hyperresponsiveness (AHR), airway smooth muscle growth, increased total airway wall area and goblet cell hyperplasia. There were no significant changes in airway wall thickening on the luminal side of the ASM bundles. Changes in the responsiveness of the lung periphery accounted for AHR whereas ASM growth affected both central and peripheral airways. The increase in AHR after multiple challenges was not significantly attenuated by budesonide (0.1mg/kg), despite its efficacy against a single challenge. Budesonide did however inhibit the increase in ASM in both central and peripheral airways, prevented the increase in goblet cells in central airways and inhibited the increased total airway wall area in the peripheral airways. The ASM and goblet cell changes were still present one week after cessation of challenges despite resolution of AHR. These observations indicate that the cause of AHR cannot simply be an increase in ASM mass.

We used both the standard single compartment model as well as the constant phase model of respiratory mechanics to examine the contribution of different lung compartments to responses to inhaled methacholine and the effects of allergen exposures on these responses. The measurement of resistance and elastance using the single compartment model has been most frequently used to assess AHR. However the constant phase model provides additional information on the site of pulmonary responses and is increasing in popularity for this reason. The constant phase model was first applied in dogs by Hantos et al. (15) but has since been used in other species including the rat (1; 24). This model allows the partitioning of responses to large airways based on changes in R_N or to the peripheral lung compartment from changes in G and H. The interpretation of changes in G and H is more complex than R_N but reflects a variety of factors, including altered rheological properties of the tissues that may be associated with hyperinflation, for example, and to losses of communicating airspaces because of airway closure (2). G and H will increase proportionately if a fraction of the lung ceases to participate in ventilation through either airway closure or alveolar

collapse (2). On the other hand, G will increase more than H if the lung becomes heterogeneous.

The AHR present after a single challenge in our data was detected by the single compartment model. The changes were detected in Ers, indicative of an alteration in the properties of the peripheral lung and consistent with this interpretation, there was also a significant change in the H parameter of the constant phase model. AHR after three Ova challenges was also associated with a predominantly peripheral lung response but was detected from the G parameter only, implying a difference in the site or mechanisms of AHR resulting from single and multiple challenges. The relatively greater change in G than H suggests that heterogeneous airway narrowing was a substantial contributor to the mechanical response. In no situation was AHR detected by R_N, suggesting that the large airways do not contribute to this phenomenon. A limitation of the interpretation of the constant phase model is that the actual airway sizes or generations corresponding to the R_N and G parameters have never been confirmed by direct techniques. Our assumption that airways larger than 1mm in perimeter should be reflected in R_N whereas smaller airways than 1mm should be captured by G has not been directly verified. However this cut-off was determined *a priori* based on previous classifications of airway size, based on knowledge of the distribution of sizes in the rat (29; 30).

A patent difference in single and multiple challenges is that the latter has an association with airway remodeling. ASM mass was increased to similar extents in central and peripheral airways by the Ova challenges whereas goblet cells, were increased to a greater extent in central airways. Increased total airway wall area also affected both central and peripheral airways. The increase in total airway wall area by far exceeds the increase in ASM area and is therefore explained by other components, such as deposition of matrix proteins (29). Despite the increase in ASM in large airways and the increased total large airway wall area, there was no alteration in the responsiveness of these airways as reflected in the R_N parameter of the constant phase model. An increase in ASM in the large airways has been described in fatal asthma and in severe non-fatal

asthma sufferers (3;6;8;28) and has been imputed to be a significant contributor to the disease severity. However it is apparent from the current study that the presence of an increase in ASM mass may not necessarily lead to AHR. Indeed the lack of association between the remodeling and AHR in the current study is reinforced by the persistence of ASM remodeling at one week after the three Ova challenges when AHR was no longer present. Recent studies have shown changes in ASM phenotype in similar animal models (26) suggesting the possibility that remodeled muscle may have reduced contractility. Some of these changes may be long-lasting; persistence of ASM remodeling at 35 days following multiple Ova challenges of the rat has been described at a time when AHR was no longer present, supporting our conclusions (23). In contrast, ASM remodeling and AHR both persist after multiple Ova challenges in the murine model (37) but the current data would suggest that the two are not necessarily causally related.

Budesonide prevented the ASM growth in all sized airways and inhibited goblet cell proliferation in the large airways. These findings suggest that ASM remodeling is relatively sensitive to steroid treatment. The sensitivity of ASM remodeling to corticosteroid treatment may reflect accompanying matrix remodeling which also occurs in the BN rat after allergen challenges (29). The inhibitory effects of corticosteroid on ASM proliferation are attenuated in the presence of collagen in human cells (4). However, collagen is deposited outside the ASM layer in the allergen-challenged rat (29) and may therefore not have a substantial influence on the sensitivity of ASM remodeling to steroid treatment. A similar study has demonstrated efficacy of ciclesonide and fluticasone against allergen-induced ASM remodeling in the BN rat (22). Steroid-mediated effects on matrix remodeling have been reported for human asthmatics (4). The increased total airway wall area that was observed in the large airways was unaffected by budesonide but total airway wall area in the peripheral airways was prevented. A dose-dependent effect of fluticasone on the attenuation of the deposition of fibronectin following prolonged repeated challenge of the BN rat has also been reported (41). Interestingly, Pini et al reported that biglycan,

collagen and decorin were prevalent in medium to large-sized airways while only biglycan was present in the peripheral airways (29). Furthermore, the amount of collagen present in the repeated Ova-challenged rats was far greater in the peripheral airways than that compared to medium to large airways (29). Thus, it is possible that the sensitivities of the matrix deposition to steroid may be dependent on not only the particular proteoglycan present but also in the amount of various proteoglycans deposited in airways of different sizes. Perhaps the matrix composition is an important underlying factor in the effectiveness of steroid in reducing total airway wall area in the peripheral airways. As there were no differences in airway wall thickening on the luminal side of the smooth muscle bundles in the repeated Ova-challenged animals, this potential aspect of remodeling cannot have contributed to AHR.

Corticosteroid treatment was used to further probe the relationship between AHR and airway remodeling. After a single challenge, the increased AHR in response to Ova was inhibited by budesonide but AHR after multiple challenges was not. The difference in treatment effects is consistent with the results of the constant phase model indicating a different spatial distribution of the AHR induced with the two different protocols. Furthermore, the data indicate that the remodeling process is an epiphenomenon and is neither necessary nor sufficient for the development of AHR. Given the uncertain relationship between AHR and remodeling we explored a panel of cytokines and chemokines found in BAL fluid 48 hrs after three Ova challenges and following budesonide. We used linear discriminant analysis (LDA) to examine the cytokines/chemokines tested as discriminating factors into the three treatments: PBS, Ova, budesonide + Ova. Kaminska et al. performed a similar analysis, the principal component analysis, to determine dominant mediators from the sputum in asthmatic individuals with fixed airflow obstruction in whom remodeling was particularly marked (18). In their study, the key variables were IL-12, IL-13 and IFN-γ in the chronically obstructed group, and IL-9, IL-17, MCP-1 and RANTES in the intermittently obstructed group (18). Interestingly, in our study, the principal discriminants of the repeated Ova-challenged group were IL-1β, IL-4, IL-13, and RANTES. IL-4

and IL-13 are well-established mediators of Th2 inflammation in allergic asthma (16; 45). IL-1β is a proinflammatory cytokine which is reportedly upregulated in BAL fluids of corticosteroid-resistant (CR) patients (14). RANTES is a chemokine for memory T lymphocytes and monocytes and has been demonstrated to increase in the BAL fluid of asthmatic patients (25). IL-9 may lead to up-regulation of mucus production (40) and is also associated with the up-regulation of the anti-apoptotic molecule Bcl-2 (35). Moreover, corticosteroids have been implicated in the apoptosis of various immune cell types and epithelial cells (9; 10). Thus, IL-9 along with the associated Bcl-2 could perhaps be protective against cell apoptosis induced by corticosteroids and thus, play a role in discriminating animals in the Budesonide + Ova group.

In conclusion, the data indicate that the site of AHR following allergen exposure is not consistent with the pattern of increased total airway wall area, airway wall thickening, remodeling of ASM and goblet cell hyperplasia. Likewise, the changes in AHR were more resistant to steroid therapy than were ASM and goblet cell remodeling. Loss of contractile phenotype which has been described for ASM in culture and *in vivo* after repeated allergen challenge in the rat could account for a lack of association between ASM mass and AHR but whether it accounts for our findings requires investigation. The cytokine/chemokine data suggest some of the important mediators that are associated with allergic inflammation and may account for AHR independently of ASM remodeling, but their relevance for airway remodeling in asthma requires further exploration.

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Grants

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Chapter 4

The potential modulation of the airway smooth muscle phenotype and the effects of the epidermal growth factor tyrosine kinase inhibitor AG1478 in a repeated allergen challenge model of asthma

4.1 **Prologue**

Growth of airway smooth muscle (ASM) occurred in large and small airways in response to repeated allergen challenge of sensitized BN rats. Despite the increase in smooth muscle (SM) in the large airways there was no evidence that these airways were hyperresponsive. These findings suggested to us the possibility that the process of proliferation resulted in de-differentiation of the ASM with the loss of contractile proteins, as has been shown in *in vitro* conditions. We wished to directly address the effects of remodeling of ASM mass on contractile phenotype. In addition because the involvement of the epithelium in the remodeling process is substantial we also examined the epithelial phenotype.

The EGFR and its family of ligands have been shown to be central to the process of remodeling through the study of *in vivo* models of asthma. To study the role of the EGFR in ASM and epithelial remodeling, and in AHR we used a selective inhibitor of EGFR tyrosine kinase, AG1478. Our objective was to examine the effects of remodeling on the expression of contractile proteins in the SM cells following hyperplastic growth induced by repeated allergen challenge and the effects of inhibiting hyperplasia on mRNA expression for muscle proteins. We used the technique of laser capture microdissection to assess smooth muscle and epithelial phenotype and we were therefore limited in our analysis of contractile and other genes of interest to their expression at the mRNA level.

4.2 **Abstract**

Introduction: Allergen challenges induce airway hyperresponsiveness (AHR) as well as an increased airway smooth muscle (ASM) mass in the sensitized rat. ASM remodeling is mediated by the epidermal growth factor receptor (EGFR). The effect of multiple allergen challenges on ASM phenotype in an *in vivo* model and the role of the EGFR in the process is not fully understood. Furthermore, how the phenotypic changes relate to AHR is also uncertain.

Aims: The aims of the study were to examine, in sensitized rats, the effects of multiple ovalbumin (Ova) challenges on ASM remodeling and phenotype, their relationship to AHR and the effects of inhibiting the EGFR on these outcomes.

Methods: Brown Norway rats sensitized with Ova were assigned to one of the following study groups: (i) Ova challenge 3x at 5 day intervals (ii) intraperitoneal AG1478 (5 mg/kg), EGFR inhibitor and Ova challenge 3x and (iii) phosphate buffered saline challenge 3x as a negative control. The constant phase model was used to assess airway responses to aerosolized methacholine (MCh) and to partition changes to large versus small airways 48 h after the final challenge and bronchoalveolar lavage (BAL) was performed. The right middle lobe was inflated with 50% OCT (optimal cutting temperature compound) before undergoing snap-freezing in liquid nitrogen for subsequent laser capture microdissection of ASM and epithelial cells. By RT-PCR, these cells were assessed for contractile protein expression and for mediators which may influence ASM phenotype, respectively.

Results: Repeated Ova challenge resulted in AHR to MCh, which the mechanical parameters (G and H) placed in the peripheral lung. There was no evidence of AHR affecting the larger, conducting airways (absence of change in RN), despite an overall increase in ASM mass and in expression of SM myosin heavy chain (sm-MHC) mRNA. Myosin light chain kinase mRNA was not affected by challenge. These effects were prevented by inhibition of EGFR. Inhibition of the EGFR (AG1478) reduced the sensitivity to MCh but not the maximal

response. EGFR inhibition attenuated airway inflammation, mainly eosinophilia.

Discussion: Repeated Ova challenges result in AHR in the peripheral airways, ASM growth in all sized airways, eosinophilic inflammation, as well as changes in sm-MHC expression. Inhibition of the EGFR attenuates inflammation, reduces ASM growth and modulates ASM phenotype.

4.3 **Introduction:** Repeated allergen challenge of the sensitized rat causes airway remodeling that is substantially mediated via the epidermal growth factor receptor (EGFR) and its axis of ligands (19; 39; 40). Along with airway hyperresponsiveness (AHR), it is considered a key feature of asthma. An increased airway smooth muscle (ASM) mass is amongst the pertinent remodeling changes. The mass of ASM may be increased potentially via a number of mechanisms: hyperplasia (7; 12; 14; 26; 30; 39; 41), hypertrophy (4; 7; 30), migration of ASM or subepithelial myofibroblasts (4), circulating fibrocytes (32) or local mesenchymal stem cells (36) and lastly, epithelial mesenchymal transition (42). Whatever the mechanism, it is not clear whether the function of remodeled ASM is normal. Hyperplasia of ASM is associated with loss of contractile proteins *in vitro*, although there are few studies addressing this issue *in vivo* (18; 20; 23; 41). ASM has been reported to be increased by hyperplasia after repeated allergen challenges in animal models (39) so that loss of contractile proteins might be expected. Some studies suggest possible downregulation of contractile proteins following allergen challenges in experimental models (18; 23) whereas studies of human asthmatic smooth muscle have failed to show such a downregulation of protein (20; 41). However, it is possible that ASM phenotype changes may only be seen in freshly remodeled ASM, which may not have been the case in studies of human tissues.

Previous work from our laboratory demonstrated decreased mRNA and protein expression for several contractile proteins normalized for ASM volume in the proximal airway tree at 48 h after multiple allergen challenges, concurrent with evidence of active proliferation of ASM (18). However, a limitation of the studies was the fact that muscle proteins and their mRNA were assessed for whole airway tissue and were not assessed in muscle directly. Data from studies of human tissues highlight that different patient populations and the manner of quantification implemented may result in discrepancies. While most studies performed on human sensitized or asthmatic tissues indicate an increase in the smooth muscle contractile protein myosin light chain kinase (MLCK) mRNA (4; 20; 21) and protein (2; 4). Woodruff et al. reported otherwise (41). Using laser

capture microdissection to capture ASM on human bronchial biopsies, they concluded that there were no significant differences in MLCK expression between asthmatic and controls (41).

Airway epithelium is also substantially remodeled in human asthma and epithelial repair is dysregulated. When epithelial surfaces are injured, the normal response is the upregulation of receptors, including members of the EGFR and its axis of ligands, that drive proliferation and repair (13). Differentiation of a fraction of the epithelial cells into goblet cells is a significant result of this remodeling process (37). Furthermore, there is a significant positive correlation between EGFR expression and hyperplastic goblet cells in asthmatic patients (38).

Epithelial remodeling is of interest both for its own sake and also for its potential role in ASM remodeling. Mediators released from the epithelium may affect ASM proliferation and phenotype. Others may have a role in inflammation. Kelly et al. reported that on laser-captured epithelial cells, TGFβ1 and plasminogen activator inhibitor-1 gene expression was upregulated 2 weeks after chronic allergen exposure in the mouse (16). In our present study, we have been interested in studying various epithelial-derived mediators including MMP-9 (a metalloproteinase) which sheds proligands for EGFR, eotaxin and RANTES, the latter two being not only chemoattractants for eosinophils but also for ASM. They may also have mitogenic actions on ASM (15).

It has been shown that EGFR expression is induced in the epithelium of Ova challenged F344 rats (37), and its expression has also been described in bronchial tissues of asthmatic patients (1; 28). A few pertinent cells that express EGFR include the epithelium (1; 27) and also the ASM (1), which is expected given that EGFR has been reported to be important for bronchial epithelial repair (28) and it is the receptor for several growth factors for ASM, namely the epidermal growth factor and heparin binding-epidermal growth factor. A few *in vivo* studies have suggested its importance in mediating AHR (19; 40). In *in vivo* asthma models, inhibiting EGFR also led to a decrease of ASM (19; 39; 40).

In the previous chapter we demonstrated that AHR affected the peripheral airways and that the large airways were remodeled but did not appear to be

hyperresponsive to methacholine despite this remodeling. Either the enhancement of the ASM force generating capacity by virtue of its increase in mass is not a determinant of large airway narrowing or a reduction in force production, presumably resulting from de-differentiation, has occurred. We wished to assess this issue directly using repeated allergen challenge to induce remodeling in the BN rat. The aim of this present study was to examine the effects of repeated allergen challenge on ASM structural and molecular remodeling leading to changes in mass and ASM phenotype. The relationship between these changes in ASM and the presence of AHR were also of interest to understanding the relationship between these two latter asthmatic features. Additionally, we were interested to observe how inflammation, ASM remodeling as well as AHR may be modulated by inhibiting the EGFR and whether the prevention of hyperplasia also prevented changes in phenotype induced by the allergen challenges.

4.4 **Methods**

Protocol for sensitization and challenge

Male Brown Norway rats (SsN substrain) (Harlan/UK) were subcutaneously sensitized with 1mg ovalbumin (Ova) along with 100 mg aluminum hydroxide dissolved in a 1mL volume of phosphate buffered saline (PBS) (39). Immediately thereafter, the rats were intraperitoneally administered $2x10^9$ *Bordetella pertussis* heat-killed bacteria (provided by T. Issekutz, Dalhousie, University, Halifax, NS, Canada). Animals under light anaesthesia were endotracheally intubated and were administered aerosol challenges while continuing to breathe spontaneously. The tip of the endotracheal tube was placed in a small plexiglass box into which aerosol was introduced from a Hudson nebulizer at 8L/min for 5 minutes, with an output of 0.15 ml/min. Rats were aerosol-challenged a total of 3 times with 5% Ova, or its control (PBS), at 5-day intervals (39) and studied 48 h after the third and final challenge. The protocols for this study were approved by the Animal Care Committee of McGill University (Montréal, QC, Canada).

Administration of EGFR inhibitor

Fourteen days after Ova sensitization as described above, one group of rats was administered 5 mg/kg AG1478 (Calbiochem, USA) in dimethylsulfoxide, (DMSO) in a volume of 700 μL while the other two groups of animals received the vehicle intraperitoneally 1 h prior to each Ova challenge.

Airway responses to methacholine challenge

Airway responses to methacholine (MCh) (Sigma Chemicals, St Louis, MO), were assessed with the linear-single compartment and the constant phase models using a small animal ventilator (Flexivent, Scireq, Montréal, QC) at 48 h after 3 Ova challenges (33). Animals were sedated and anesthetized with xylazine (10 mg/kg) and pentobarbital (35 mg/kg) respectively and paralysis was induced with 1mg/kg pancuronium bromide intraperitoneally (Sandoz Canada Inc, QC). The animals were placed on a heating pad and their temperature was monitored to ensure that it remained close to 36^{o}C. A positive end-expiratory pressure was maintained between 2 and 2.5 cmH_2O and the animals were ventilated at a tidal volume of 8 mL/kg and a breathing frequency of 90 breaths/minute. The constant phase model was used to partition mechanical responses within the lungs. The latter model fits the data to an equation that has four parameters; R_N (Newtonian "airway" resistance), I_{aw} (airway inertance), dominated by the mass of gas in the central airways, and impedance of tissues accounted for by both G and H. G (tissue damping) is closely associated to peripheral airway and tissue resistance and reflects energy dissipation in the lung tissues, H is tissue elastance and reflects energy storage in the tissues (11).

Bronchoalveolar lavage (BAL) fluid and assessment of inflammation

BAL fluid (5 x 5 mL aliquots of PBS) was drawn from the rats subsequently following the assessment of airway responses. Cells were pelleted upon centrifugation from all the aliquots and re-suspended in PBS to perform cell counts. Cytospins were then prepared using a cytocentrifuge (Shandon

Cytospin® 4 cytocentrifuge, Thermo Scientific, Waltham, MA) and were subsequently stained with Diff-Quik® Stain Set (Dade Behring, Newark, DE).

Cytokine/Chemokine Analysis

To assess the relationship of AHR and remodeling to inflammatory mediators we performed a multiplex cytokine analysis on the first 5 mL of BAL samples retrieved at the 48 h time point in the following groups using the MSD (Meso Scale Discovery, Gaitherburg, MD, USA) technology. The following cytokines/chemokines were included in the analysis: GRO/KC, IFN ɣ , TNF-α, IL-1β, IL-4, IL-5, and IL-13.

Histology and morphometry

For the assessment of remodeling, the left lung lobes were fixed overnight in formalin at a pressure of 25 cmH_2O and were paraffin-embedded. Histological slides were prepared from 5 μm mid-sagittal and para-hilar sections to sample medium and small airways and large airways, respectively. Tissue sections were stained for smooth muscle-specific α -actin (α -SMA) to assess any increase in ASM mass. Mass of ASM was estimated from the area of smooth muscle-specific α -actin immunoreactivity as previously described (29; 39). Isotype controls were performed and showed no staining, confirming the specificity of smooth muscle-specific α -actin staining. Airways $\geq$1mm basement membrane perimeter (P_{BM}) were considered to be medium and/or large airways and <1mm P_{BM} as small airways (29). Airways whose ratio of maximal to minimal internal diameter was equal to or greater than 2 were not included in the analyses.

In brief, sections were stained with a mouse monoclonal antibody to α-SMA (clone 1A4; Sigma-Aldrich Co.) as well as a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). The signal was developed with Vector® Red. The area of ASM was traced using a camera lucida side arm microscope attachment and was digitized with commercial software (SigmaScan Pro 5, Ashburn, VA). The area of ASM was normalized for airway size by dividing by the square of the P_{BM}. Angiogenesis was also assessed with a count of number of

blood vessels normalized for airway size by dividing by the square of the P_{BM} in both the more central versus the more peripheral airways.

Laser capture microdissection (LCM) for epithelial and airway smooth muscle cells

The right middle lobe was inflated with 50% OCT (optimal cutting temperature compound) and fixed with OCT before being snap-frozen in liquid nitrogen for subsequent laser capture microdissection (Arcturus, Veritas, USA). Seven μm-thick sections were prepared in a cryostat (-20^{o}C) in a RNAse-free environment. The sections were stained with hematoxylin and eosin following protocols developed for LCM. LCM was performed using macro caps to select for epithelial cells. High sensitivity caps were used to select for airway SM cells. Once the cells were captured within 1-1.5 h after the staining, 4 μL of Side-step lysis buffer (Stratagene, CA, USA), which allows the lysis and the preservation of mRNA of cells and subsequent reverse transcription side-stepping the mRNA extraction step, was used to extract the cells. The cells were placed in 500 μL tubes and were vortexed for 1 minute allowing the cells to be lysed. The samples were then stored at -80^{o}C until the reverse transcription step.

Reverse transcription and RT-PCR

The Agilent/Stratagene kit for reverse transcription (Santa Clara, CA, USA) was used for the laser captured cells. Epithelial cells were subsequently assessed for TGFβ1, eotaxin and RANTES, MMP-9, the neutrophilic chemoattractant Gro-α. ASM cells were assessed for SM contractile proteins, myosin heavy chain (MHC), myosin light chain kinase in addition to vimentin and CD90. All genes of interest were normalized to S9, a constitutively-expressed, housekeeping gene.

Statistical analysis

Data are presented as mean ± SEM. The statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA). For AHR data, repeated measures ANOVA followed by Bonferroni correction was

performed. For ASM data and MSD cytokine/chemokine analysis, BAL fluid inflammatory cell count and gene expression data, a one-way ANOVA was performed followed by Newman-Keuls post-hoc was performed. A p-value of <0.05 was considered significant.

4.5 **Results**

Bronchoalveolar lavage fluid (BALF)

The total number of BALF cells was significantly increased in the Ova-challenged ($p<0.01$) and AG-treated Ova-challenged ($p<0.05$) when compared to the PBS-challenged, negative control animals. Eosinophils were increased in Ova-challenged animals ($p<0.001$) and were also higher in AG-treated Ova-challenged animals ($p<0.001$) compared to PBS-challenged animals. However, AG-treated Ova-challenged animals had a markedly lower number of eosinophils when compared to Ova-challenged animals ($p<0.05$). (Figure 1)

MSD analysis for BALF

There were no significant differences in any of the mediators assessed in the BALF despite increased mean values in the Ova-challenged compared to controls which were lower in the AG-treated, Ova-challenged animals for IL-4 and IL-13 (n=5-6). (Data not shown)

Allergen-induced airway hyperresponsivness to MCh; total respiratory system AHR and constant phase parameters

There was a significant increase in the Ova-challenged animals at 16, 32, and 64 mg/mL MCh in the total respiratory system resistance ($p<0.05$, $p<0.01$, $p<0.05$ respectively) and elastance ($p<0.05$, $p<0.001$, $p<0.01$ respectively). EGFR inhibition prevented total respiratory system elastance at 16, 32 and 64 mg/mL ($p<0.05$, $p<0.001$, $p<0.01$ respectively). There were no significant differences in RN, reflecting the responses of large, conducting airways. G and H, reflecting the peripheral responses of the lung, were significantly higher in Ova compared to PBS-challenged animals at a submaximal dose of MCh (32mg/mL) ($p<0.01$). EGFR inhibition inhibited this increase in airway response ($p<0.05$). (Figure 2)

Morphometric assessment of ASM mass

There was a significant increase in the mass of ASM in the Ova-challenged animals in the large ($p<0.05$, $p<0.01$) and small airways ($p<0.05$). The increased mass in both the large and small airways is significantly inhibited by the EGFR inhibitor, AG1478. (Figure 3)

Angiogenesis

There was no significant increase in the number of vessels in the large airways. However, the number of blood vessels in the AG-treated, Ova-challenged animals was significantly higher than the PBS-challenged and the Ova-challenged animals in the peripheral airways ($p<0.05$). Furthermore, there were no significant differences in the vascular smooth muscle areas in the Ova-challenged animals compared to controls for either the large or small airways. (Figure 4)

Gene expression in laser-captured ASM cells

There was a significant increase in the expression of smooth muscle myosin heavy chain (MHC) in the Ova-challenged animals ($p<0.05$) that was not present in the animals in which the EGFR was inhibited ($p<0.001$). There was no difference in total myosin light chain kinase (MLCK) amongst the groups. There were also no significant differences in the expression of vimentin. However, CD90, a marker highly expressed in mesenchymal stem cells was decreased in the Ova-challenged animals ($p=0.05$) and this decreased was prevented with EGFR inhibition ($p=0.07$, when comparing Ova to AG-treated, Ova-challenged animals). All RT-PCRs were normalized to the expression of S9, a housekeeping gene. (Figure 5)

Gene expression in laser-captured epithelium

We examined the expression of eotaxin and RANTES, chemoattractants for eosinophils and found no differences amongst the three study groups. We also examined CLCA3, which is closely associated to increased Muc5ac (known to be

involved with subsequent goblet cell expression). Other genes tested included TGF-β1, matrix metalloproteinase-9 (MMP-9), Gro-α and IL-13. There were no significant differences to report amongst the groups for any of these aforementioned genes. (Figure 6)

Figure 1: BALF: Measure of inflammation

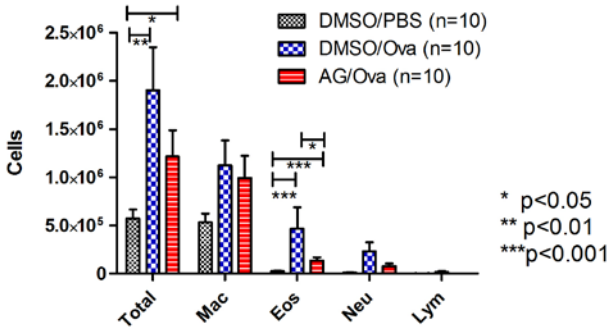
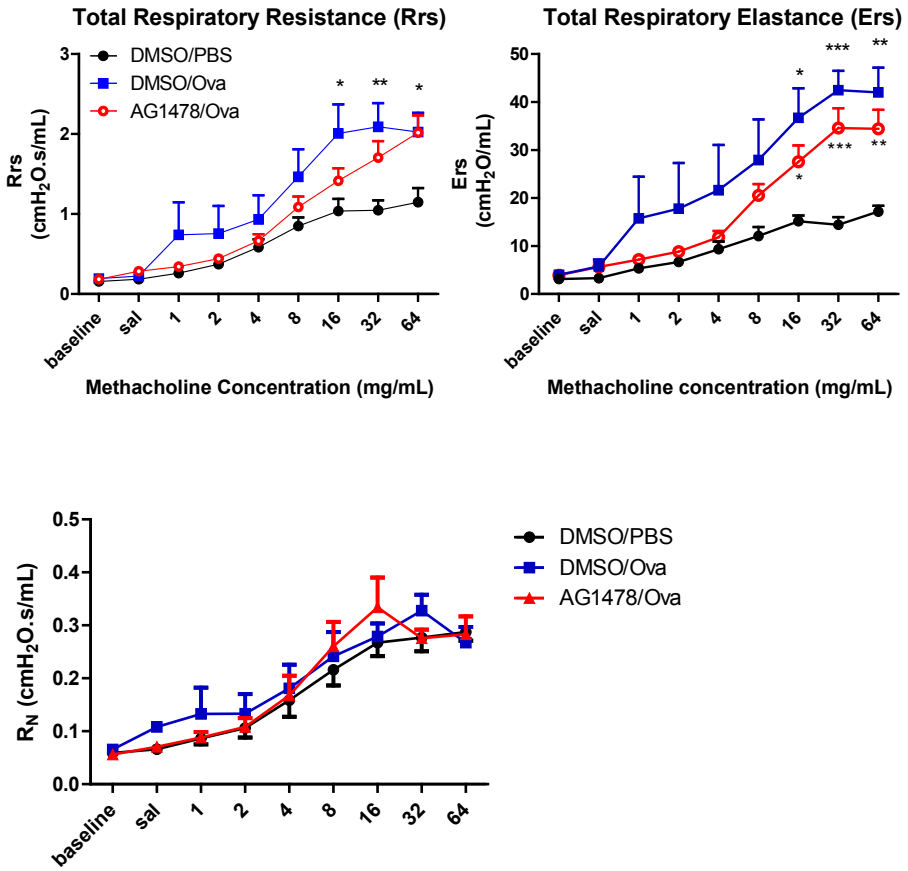


Figure 2: Total respiratory system AHR and constant phase parameters

***p<0.001, **p<0.01, *p<0.05 (DMSO/Ova versus DMSO/PBS),

(AG/OVA versus DMSO/Ova), n=7-10



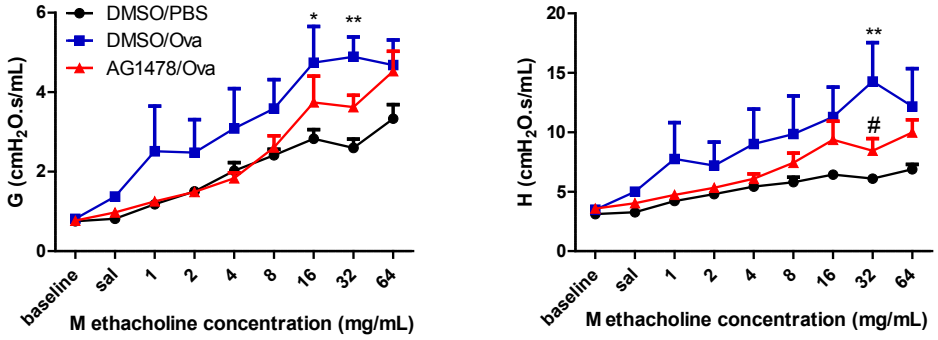
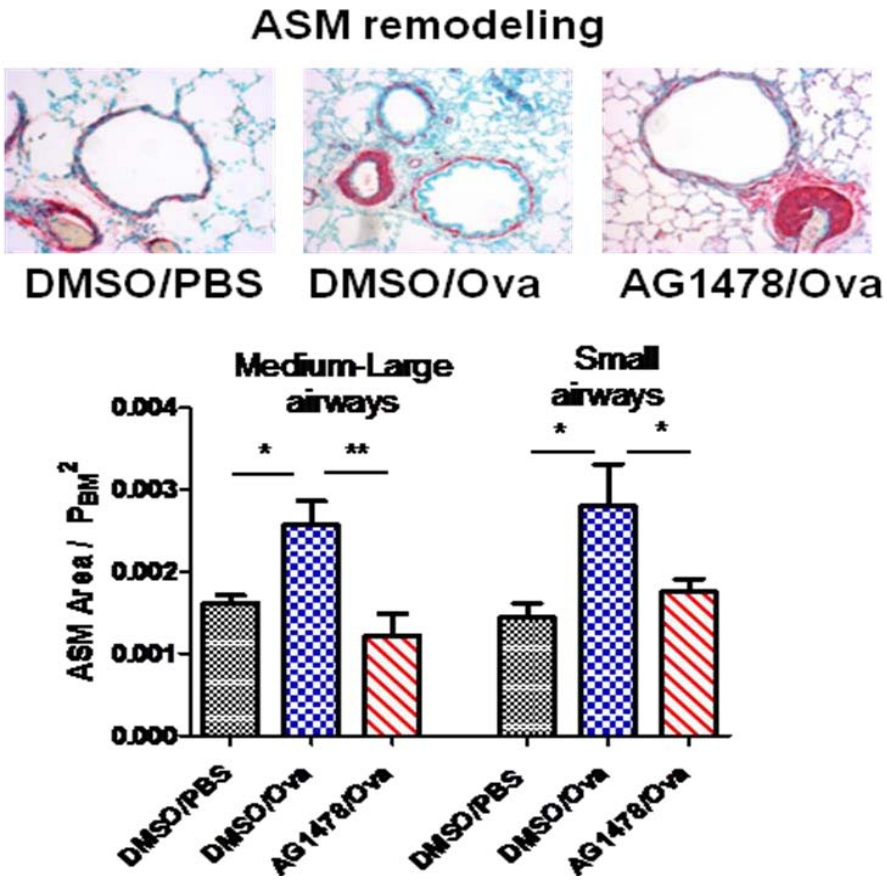


Figure 3:



*p<0.05, **, p<0.01

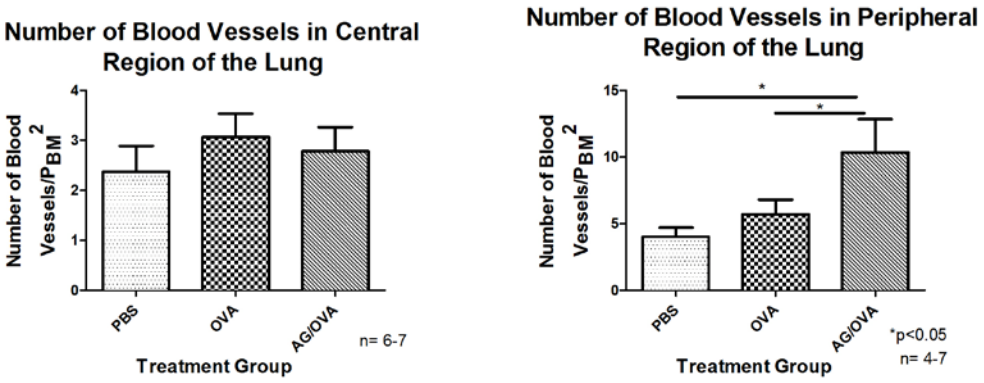
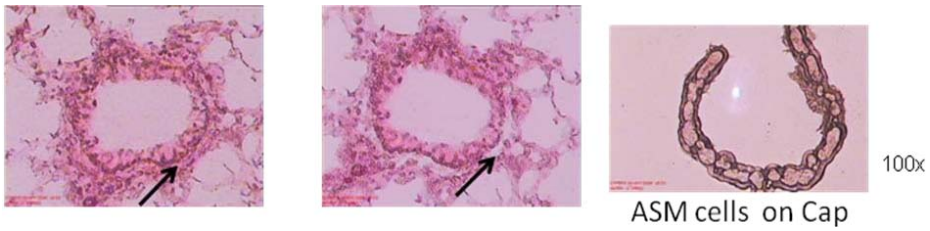
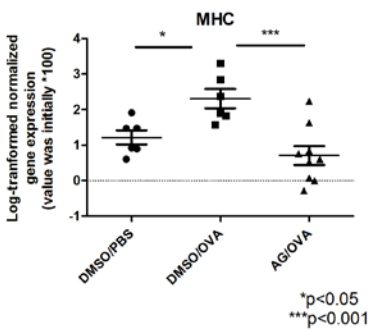
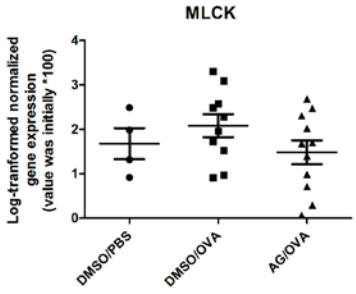


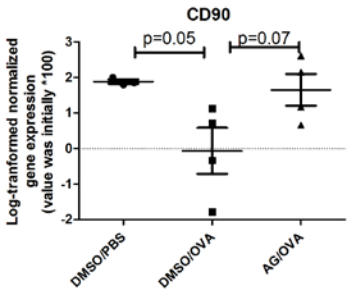
Figure 4: Angiogenesis and vascular smooth muscle area

Figure 5: Gene expression for LCM-captured ASM









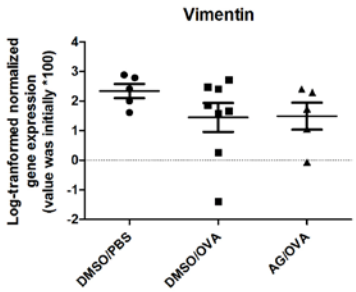
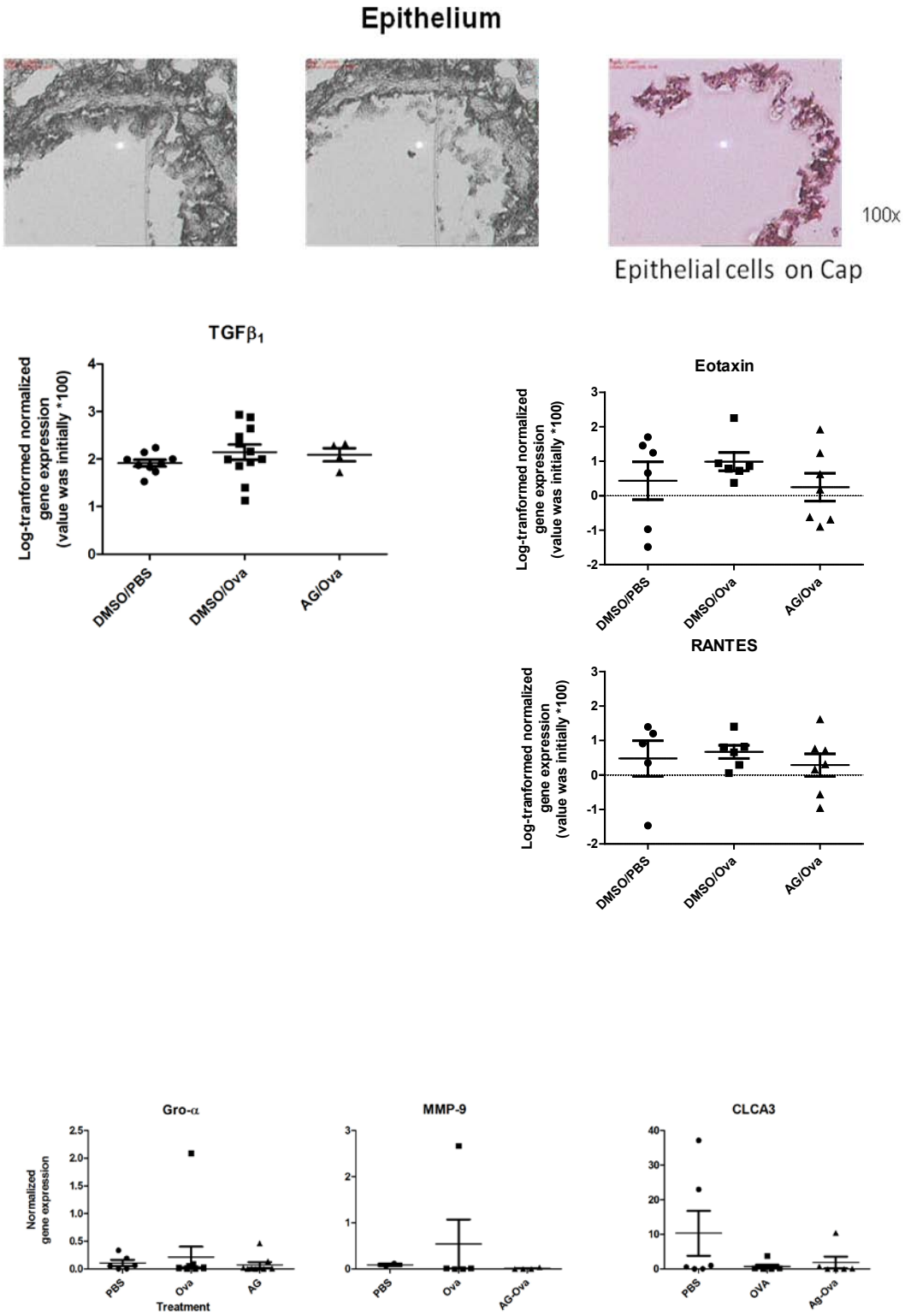
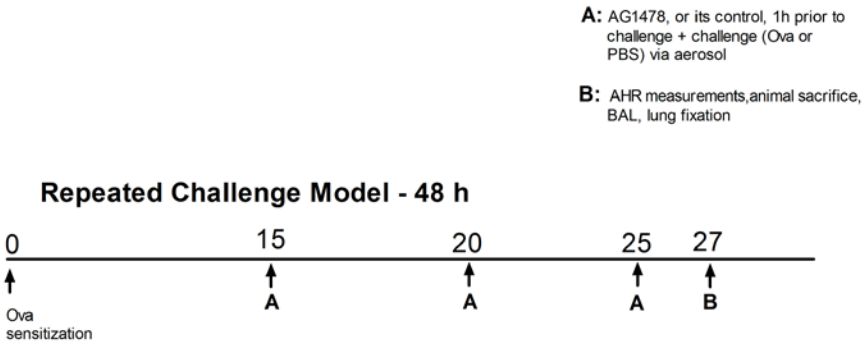


Figure 6: Gene expression from epithelial-derived mediators after laser capture microdissection.



Supplementary figure 1.



4.6 **Discussion**

Repeated Ova challenge led to AHR in the peripheral lung compartment but did not affect the responsiveness of large airways. Remodeling affected both large and small airways, causing an increase in ASM mass. Despite the increase in tissue mass and in the expression of one of the important contractile proteins, MHC in ASM large airway responsiveness was not increased. The increase in ASM mass and in MHC expression was attenuated by inhibiting the EGFR, confirming the role of this receptor in mediating the growth of ASM. Inflammation was also observed in the allergen-challenged animals and this was also reduced by inhibiting the EGFR. Repeated allergen challenges in the sensitized BN rat result in AHR. We have shown recently that this AHR is located in the periphery of the lung based on the evaluation of respiratory mechanics using the constant phase model (33). Our present data are in concordance with these earlier findings. The mechanisms of AHR have not been definitively elucidated although it seems likely that there are different stimulus-dependent pathways that may lead to the same excessive narrowing of the airways. It is not clear how airway remodelling contributes to AHR. In the current study repeated allergen challenges led to increased ASM growth in all sized airways. It is possible therefore that remodeling of the small airways may have contributed to AHR. However a similar argument cannot be made for the remodelling of the large airways that did not have any obvious effect on respiratory mechanics. We did not detect an increase in either blood vessel density or in vascular smooth muscle area in the Ova-challenged animals compared to controls. Concurrent EGFR inhibition reduced allergen-induced ASM growth as reported earlier (39). Unexpectedly, EGFR inhibition led to a greater number of blood vessels compared to the both PBS and Ova-challenged animals.

Previous studies have shown that increased ASM mass is attributable to hyperplasia (25; 29; 39). Based on cell culture models we would have expected intrinsic changes associated with proliferative and secretory characteristics. To address the possible alteration in ASM phenotype we examined the expression of

proteins in the contractile pathway, myosin heavy chain and MLCK. Previously alterations in contractile protein expression have been related to clinical outcomes; sm-MHC, sm-α-actin, and desmin expression levels have been shown to correlate with methacholine responsiveness in asthmatic subjects (20; 34). PC20 for methacholine was inversely related to the expression of sm-α-actin, desmin, and elastin (34). In addition, FEV1 (% predicted) was positively related and deep inspiration-induced bronchodilation inversely related to desmin, MLCK, and calponin expression (34). Although we observed an increase total sm-MHC after repeated allergen challenges, EGFR inhibition inhibited the increase in sm-MHC, linking the effects of the receptor to both hyperplasia and to increase in contractile phenotype.

We did not however observe differences in the gene expression of total myosin light chain kinase. Our results are in accordance with a human study, which also made the use of the laser capture microdissection (LCM) technique to dissect SM from biopsies and reported no differences in MLCK expression between asthmatic and control bronchial biopsies (41). These findings are dissimilar to findings where there an up-regulation of MLCK mRNA in mild asthmatic bronchial biopsies has been reported (20). Using LCM in our study allowed the added advantage of assessing gene expression in a homogenous tissue population.

We also assessed ASM for CD90 expression. CD90 is a marker which is highly expressed in mesenchymal stem cells as well as myofibroblasts. It is less abundant in fibroblasts. We anticipated that CD90 expression might be increased after allergen challenges if primitive cells were recruited as part of the remodeling process. Contrary to our expectations we found a reduction in CD90 in the Ova-challenged animals. Perhaps an increase in the differentiation state of ASM may have been associated with such a change. We currently have no explanation for the finding. In the same vein, we also assessed gene expression of vimentin in the ASM as this is also differentiation marker for fibroblasts. There were no notable differences amongst the groups.
Since the epithelium is a probable source of EGFR ligands and the proforms of

these molecules may be cleaved at the membrane by metalloproteinases such as MMP-9, we assessed the expression of this particular MMP. There is evidence for epithelial influence on ASM phenotype via a MMP-9 dependent mechanism (22) although earlier reports suggested that the role for MMPs in smooth muscle proliferation was unclear (3; 9; 10). We thus assessed the gene expression of epithelial-derived MMP-9 but we observed no changes in gene expression amongst the groups. We also assessed epithelial-derived TGFβ1 which has been reported in an *in vivo* asthma model to be up-regulated but we found no differences amongst the groups, arguing that epithelial-derived TGF β1 is not responsible for the increase in ASM mass, as had been previously proposed (16). TGF β1 is a profibrotic cytokine and has also been implicated in cellular hypertrophy *in vitro* (8) and and *in vitro* it has also been shown to induce ASM proliferation (5). We assessed IL-13 as well as a potential modulator of ASM phenotype and the cytokine most strongly associated with the development of AHR. Very few samples expressed IL-13 and thus, there were no differences observed. Gro- α is an important neutrophilic chemokine and we assessed its expression in the epithelium but there were no differences amongst the groups.

In an attempt to demonstrate epithelial cell metaplasia to goblet cells, we assessed CLCA3, a calcium-activated chloride channel marker, commonly considered as a mucus gene (24; 43). Despite earlier data where there was a confirmation of goblet cell production as assessed by Periodic Schiff staining in this BN rat model (33), we did not find differences amongst the three study groups for CLCA3. These data support findings where gob-5 (also known as CLCA3) was shown not to be important in mucus overproduction in a preclinical study of allergic asthma in a murine model (31). Other mucus genes might be of importance to evaluate. It is also possible that the timing of the harvesting of tissues two days after the last challenge may not be optimal to assess mucus genes.

Ova challenge induced inflammation, as observed in increased total cell counts and eosinophils in the BALF. EGFR inhibition led to a decrease in both total cell counts and eosinophils. These observations suggested to us the

possibility of an effect on EGFR activation during allergen challenge on chemokine expression. Gene expression data from laser-captured cells indicated that epithelial-derived eosinophilic chemotactic factors, RANTES and eotaxin may not be responsible for the reduction of the eosinophilic inflammation by EGFR inhibition. However, we did not explore the ASM-derived chemotactic factors as being possible contributors along with the notion that there may be synergy with other mediators implicated in eosinophil recruitment *in vivo* (17) including 5-lipoxygenase product, 5-oxo-6,8, 11,14-eicosatetraenoic acid (5-oxo-ETE) (35) and leukotriene B4 (LTB4) (6). Furthermore, we did not find marked differences amongst the groups for the protein assessment in the brochoalveolarlavage of GRO/KC, IFN ɣ , TNF-α, IL-1β, IL-4, IL-5, and IL-13. The expression of these cytokines and chemokines may be time-dependent after allergen challenge and the time of harvest of BALF may have been too late to detect significant changes.

In summary, we have explored the relationships among ASM remodeling, AHR and ASM gene expression. We are left with a paradox as our data indicate an increase in ASM mass in large airways, an increase in sm-MHC expression but no modulation of other contractile proteins but a lack of change in large airway contractile responses to aerosolized methacholine. Our findings do not preclude a role for ASM remodeling in the AHR in the peripheral lung. Undoubtedly there is a myriad of events occurring simultaneously that could synergistically be contributing to muscle contractility and perhaps, merely mechanical properties of the ASM or changes in gene expression of muscle are not sufficient to do so. Assessment of expression of related mediators including myosin light chain phosphatase (MLCP) that inhibits the activation of MLCK, CPI-17 (the inhibitor protein for MLCP) and rhoK may help to more fully understand the consequences of ASM remodeling for its phenotypic characteristics.

Table 1 : **Primer Sequences**

S9 Forward primer	AGGATTTCTTGGAGAGAAGGCTGC
S9 Reverse primer	CTTCTGAGAGTCCAGGCGAACAAT
Myosin heavy chain (MHC) Forward primer	CCAATCCTGGAGGCTTTCG
Myosin heavy chain (MHC) Reverse primer	CTCTCGTCCCTAGCATGTC
Myosin light chain kinase (MLCK) Forward primer	ACATCCGTCAGGAGATCAGC
Myosin light chain kinase (MLCK) Reverse primer	CACTCCCGCTCTGTTAGCTC
vimentin Forward primer	AACACTCCTGATTAAGACGGTTG
vimentin Reverse primer	TCATCGTGGTGCTGAGAAGT
CD90 Forward primer	CCACAAGCTCCAATAAAACTATCAA
CD90 Reverse primer	AGCAGCCAGGAAGTGTTTTG
TGFβ Forward primer	CCTGGAAAGGGCTCAACAC
TGFβ Reverse primer	CAGTTCTTCTCTGTGGAGCTGA
Eotaxin Forward primer	CACGGCCACTTCCTTCAC
Eotaxin Reverse primer	TGGGGATCTTCTTACTGGTCA
RANTES Forward primer	CTCACCGTCATCCTCGTTG
RANTES Reverse primer	GAGTGGTGTCCGAGCCATA
CLCA3 Forward primer	TCTCCTCAGGAAATGCAGCTA

CLCA3 Reverse primer	GGAGATTAACTCCTCTGCTCTCC
MMP-9 Forward primer	CCTCTGCATGAAGACGACATAA
MMP-9 Reverse primer	GGTCAGGTTTAGAGCCACGA
GRO-α Forward primer	CACACTCCAACAGAGCACCA
GRO- α Reverse primer	TGACAGCGCAGCTCATTG
IL-13 Forward primer	GGCCCTCAGGGAGCTTAT
IL-13 Reverse primer	GCTGTTGCACAGGGAAGTCT

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Discussion

5.1 **Discussion**

The themes addressed in this doctoral thesis are the relationships between ASM growth, ASM phenotype and the sites and magnitude of airway narrowing following allergen sensitization and challenge and subsequent methacholine (MCh) challenge. The aims of the thesis were to elucidate the consequences of allergen sensitization and challenge for airway remodeling and the role of airway remodeling in airway hyperresponsiveness. In chapter 2 we assessed the early allergic response (EAR) which occurs within minutes after an allergen challenge. We determined, using a structure-function analysis, that the early response to inhalational challenge was initiated in the larger airways and spread to the peripheral lung over the ensuing minutes. We observed that natural surfactant, rich in surfactant protein (SP)-B and lacking the commonly associated immunomodulatory proteins A and D, has potentially protective effects in inhibiting allergen-induced bronchoconstriction. Not only did surfactant inhibit airway narrowing, an effect potentially attributable to its physical properties that reduce surface tension at the air-liquid interface in the airways, but its inhibition of the release of mast cell products indicates that it has immunomodulatory effects, which has important implications in the context of the EAR. These data are consistent with an earlier report that proposed that surfactant protein B has anti-inflammatory effects (20). It was reported that surfactant protein B successfully inhibited lipolysaccharide (LPS)-induced nitric oxide production by rat alveolar macrophages via a reduction in the protein levels of nitric oxide synthase (20). In 2001, another study reported immunomodulatory effects of exogenous surfactant (Survanta®) which is abundant in SP-B and SP-C, but not SP-A and SP-D. Survanta® (Ross Products Division, Abbot Laboratories, Columbus, OH) is an exogenous commercially-available, bovine surfactant normally used for intratracheal administration in infants with respiratory distress syndrome (29). LPS or bacterial induced tumor-necrosis factor-α (TNF-α) or luminol-enhanced chemiluminescence (LCL), a measure of respiratory burst, released from murine macrophages was inhibited by this particular exogenous surfactant (29). In summary, our data highlight the transient large airway

repsonse in the EAR which becomes more prevalent in the periphery over time and the potential of surfactant as a beneficial therapeutic candidate to counter bronchoconstriction.

Our data further suggest that ASM remodeling and increases in total airway wall area induced by repeated allergen challenges involve the entire bronchial tree whereas AHR reflects alterations in the lung periphery. There were, however, no significant observable differences in inner airway wall area, from the inner aspect of the ASM bundles to the surface of the epithelium. Changes in airway thickness in this compartment have been invoked as a cause of AHR (11; 23). Indeed an increase in epithelial thickness alone has been shown in allergen challenged mice and proposed as a part of the mechanism of AHR (33). Furthermore, AHR with allergic airway inflammation has been explained by exaggerated closure of peripheral airways (33). Increases in total airway wall area in the peripheral airways are inhibited by low dose corticosteroid, a first line anti-inflammatory drug used to control human asthma. Meanwhile, prevention of AHR induced by repeated allergen challenges requires higher doses of corticosteroid than are required to inhibit remodeling. Repeated ovalbumin (Ova) challenge also leads to inflammation as well as increased expression of the smooth muscle myosin heavy chain, a smooth muscle contractile protein, which are attenuated by inhibiting the epidermal growth factor receptor (EGFR) using AG1478 which specifically targets its phosphorylation. In summary, after a single challenge and repeated allergen challenges, features commonly associated with the EAR and asthma are observed respectively and the successful intervention by a variety of means; exogenous, natural surfactant (Infasurf®), corticosteroid (budesonide), and EGFR inhibition (via AG1478) help to further delineate the varying sensitivities and possible mechanisms involved with bronchoconstriction, AHR, airway remodeling and phenotypic modulation.

However, as all our studies relied heavily on the constant phase model and its interpretation in the assessment of airway function, as described in chapter

1 we confirmed the partitioning of the lung responses by the parameters of the constant phase model into central and peripheral lung components. To do this we assessed airway narrowing directly on histological sections of large and peripheral airways using the snap freezing technique and subsequently, comparing the sites of airway narrowing to that implied from mechanics. We concluded that while R_N assesses larger, conducting airways, H, being a peripheral component, assesses more peripheral airways but surprisingly, G, despite being regarded as a parameter sensitive only to changes in the peripheral lung compartment, may be coupled to R_N and is sensitive to changes in both central and peripheral airways. Our data also indicate that heterogeneity of airway narrowing is more apparent over time after an allergen challenge.

In chapter 2 we also discussed how exposure to Ova, the experimental allergen driving our animal model, triggered a significant early airway response (EAR), reflecting a significant degree of bronchoconstriction. The peak of the EAR occurred at ~ 3 minutes after the Ova challenge. We ruled out that the mere distribution of mast cells and airway smooth muscle mass across varying airway sizes were the sole determinants of the site of airway narrowing during the EAR. We hypothesized that surface tension may play an important role in modulating EAR and thus decided to use a naturally occurring surfactant which was collectin-free and expected to inhibit surface tension in our study as we explored possible determinants of the EAR. Infasurf®, a natural, calf surfactant, which does not contain the surfactant proteins A and D, the commonly known immunomodulatory proteins, decreased the observable peak magnitude of the EAR as indicated by the R_N parameter. It was administered via ultrasonic nebulization prior to the Ova challenge. This is the first ever reported use of constant phase parameters to assess the EAR with a comparison to airway narrowing documented by histological techniques. Interestingly, surfactant also inhibited release of the mast-cell mediators, cysteinyl leukotrienes and amphiregulin. This portion of the study is novel in that the inhibition of mast cell mediators in the EAR by exogenous, collectin-free surfactant administration has

never been reported. Natural surfactant has potentially protective effects in inhibiting airway obstruction and its effects may involve immunomodulation in addition to the commonly known physical effects. However a study of the effects on mast cell activation by IgE cross-linking *ex vivo* did not reveal any inhibition of mast cells by surfactant. It thus appears that surfactant has effects on mast cell activation that are indirect.

In chapter 3, we investigated the relationship between allergen-induced remodeling and AHR using budesonide, a corticosteroid commonly used in regulating asthma and an effective anti-inflammatory agent, as a probe to further delineate any potential differences between the two asthmatic features. After repeated allergen challenges, there was a significant increase in ASM in all airways and in goblet cells in the large airways, changes which were inhibited by low dose budesonide (0.1mg/kg). Total airway wall area was increased in airways of all sizes but the change in area was only inhibited in the peripheral airways. A detailed analysis of the site of AHR using the constant phase model indicated that AHR was attributable to excessive responses of the small airways whereas responsiveness of the large airways was unchanged. Low dose budesonide did not inhibit AHR after repeated challenge but it did inhibit remodeling. However, low dose budesonide was effective in inhibiting AHR after a single challenge, suggesting potentially different mechanisms of AHR induced by a single challenge from that induced by multiple challenges. Furthermore the data indicate that the sensitivities to the budesonide dose required to inhibit the development of AHR and airway remodeling are different. These data highlight how different mechanisms are at play in both of these characteristics of asthma. ASM and goblet cell changes were still present one week after cessation of challenges despite resolution of AHR. These observations indicate that the cause of AHR cannot simply be an increase in ASM mass. An important consideration as to how long AHR or airway remodeling lasts in a given model may depend on but may not be limited to differences in choice of the animal model, delivery and time course of allergen delivery, but also may depend on important

considerations as to how much exposure there is, to triggers of innate immunity such as contamination by LPS in the allergen of choice and how that may alter the development of the subsequent responses. Using LPS contamination in Ova as an example, in the BN rat model of asthma, after multiple allergen challenges in the sensitized rat, LPS contamination has been observed to contribute to the influx of inflammatory cells, macrophages and neutrophils, to the airways, while not affecting Th1 and Th2 cytokines and airway remodeling (12).

In chapter 4, we pursued the study of ASM phenotype in relation to AHR and of the effects of inhibiting the epidermal growth factor receptor (EGFR), the dominant pathway for airway remodeling. Repeated allergen challenges upregulated the expression of smooth muscle myosin heavy chain (sm-MHC), a contractile protein. Inhibition of the EGFR by AG1478 modulated the increase in smMHC, bringing it back close to control levels, as it did to the mass of ASM. The EGFR may contribute to ASM phenotype. It has been shown that the proliferation of ASM is associated with a loss of contractile protein (21). Indeed studies from our own laboratory were consistent with the same idea (17). A unique feature of our study was that since it was using an *in vivo* model we also were able to provide the corresponding physiology as well as laser microdissection and capture for the investigation of the effects of the process of remodeling on specific cell types. We were particularly interested in ASM and epithelial cells and wished to explore potential phenotypic modulators for these cells. We probed the model with an EGFR inhibitor which reduced the increased ASM growth observed in Ova-challenged animals in all airway sizes. The fact that EGFR inhibition was effective in inhibiting ASM growth regardless of airway size was an additional insight into the structural changes occurring to a previous study our lab published relatively recently (30). Furthermore, AG1478 altered the concentration response curve to challenge with MCh, reducing allergen induced AHR at low but not high concentrations of MCh. The inhibition affected the parameters that captured peripheral airway responses. There were also no differences amongst groups in the Newtonian resistance (R_N) which is

indicative of responses in the larger, conducting airways, despite a substantial increase in muscle in these airways. We are thus left with an apparent paradox whereby there is an increase in ASM mass in large airways but an increase in sm-MHC and no obvious down-regulation of other proteins in the pathway to contraction. It is possible that changes in the mechanical properties of the ASM are not key determinants of responses of large airways or that the changes in muscle protein are not sufficient to increase muscle contractility.

AG1478 administration to allergen challenged animals also inhibited inflammation, as evidenced by the significant reduction in the number of eosinophils in BALF. Similar observations have been made in mice and leukotrienes have been implicated in mediating the Ova-induced changes via EGFR (32).

We chose the Brown Norway rat to explore our questions because it is a well-characterized model of allergic asthma. In addition it has an advantage over murine models in that it has airway architecture which is closer to that of larger mammals with a bronchial circulation. Other rat strains that have been used in studies of airway physiology are the Lewis and Fisher rats. Their genetic make-up is such that even without allergen exposure, Lewis rats are commonly known to be normo-responsive to airway challenge with MCh while Fisher rats are hyperresponsive to MCh (4). Fisher rats have more ASM than Lewis rats, a trait that has been implicated in the airway responsiveness (4) as well as a reduced level and a slower rate of spontaneous relaxation of tracheal smooth muscle (1). All these rat strains have their particular places in the evaluation of ASM phenotype in experimental asthma. However, there are still many questions yet to be answered concerning airway remodeling in animals. The mechanisms that have emerged from animal studies have not as yet been cross-checked for their pertinence for human airway remodeling. Thus far, there are insufficient links of any specific component of the inflammatory response to human remodeling in any definitive fashion. It seems likely that airway remodeling results from cycles

of repeated injury and repair or perhaps from persistent inflammation. More importantly, the reversal of remodeling is an important therapeutic objective because it is a potentially disease-modifying strategy.

In our studies, a common underlying thread was assessing a present-day clinical therapy for asthma (budesonide, a corticosteroid) and potential targets for drug development for asthma (EGFR and surfactant) in delineating structure-function relationships. Thus, mechanical assessments versus assessments of structural and histological modulation in both multiple allergen-challenged asthma and single-challenged EAR *in vivo* models were studied. For a multiple challenged *in vivo* model of asthma, corticosteroids can be effective at the level of sensitization; human studies with asthmatics have indicated a correlation between the reduction in number and function of airway dendritic cells and clinical efficacy (5; 9; 22). While we did not study the role of corticosteroids at the stage of sensitization, we did administer steroid prior to every allergen challenge, hoping it would provide better insight into the varying sensitivities for the amelioration of AHR and airway remodeling. We concluded that AHR and airway remodelling are not tightly coupled phenomena given that budesonide did not inhibit AHR in doses that did inhibit ASM growth in all airways and in an *in vivo* asthma model, AHR wanes earlier than airway remodelling.

The role of EGFR in mediating AHR and airway remodelling has been explored prior to our study (30; 32) but its role in potentially modulating ASM phenotype or with the intended partitioning of both AHR and remodelling responses is a novel undertaking. Our data are in concordance with our corticosteroid data, suggesting that the effects of EGFR inhibition on AHR and ASM are not coupled and interestingly, its effects on ASM mass reduction, like budesonide, affect all-sized airways. Both the AG1478 compound and budesonide were administered concurrently to allergen challenges. As previous *in vivo* studies with corticosteroids indicate, corticosteroid treatment after

repeated allergen challenge treatment is less effective in inhibiting airway remodelling (8; 31).

The interactions of corticosteroids and EGFR, given that EGFR is a receptor for ASM growth factors and also that it mediates airway remodeling and AHR (18; 30; 32) are of investigative interest as well. It is of added interest given that we studied both independently in the same BN rat model as we worked our way to understand the relationship between AHR and airway remodeling. It was reported in 2000 that EGFR immunoreactivity in the epithelium was unaffected by corticosteroid treatment (24). It was later reported that an increase in phosphotyrosine levels in the severe asthmatic bronchial epithelium were unaffected before or after 8 weeks with budesonide (7). There was a partial, yet transient, suppression of EGFR phosphorylation with salbutamol, a β2-adrenergic agonist, but dexamethasone had no effect (7). These data from human biopsies provide little in terms of noteworthy effects of corticosteroids on the EGFR.

In conclusion, our data along with previously published data indicate that surfactant, commonly used for treating respiratory distress syndrome or acute lung injury, may have an important role in reducing EAR responses and thus, possibly in asthma. It may be worthwhile for future *in vivo* studies to be designed to continue to incorporate a combination of the therapies that have shown solid potential on their own. Such a study would be similar to the one performed by Henderson et al. (2006) where a corticosteroid (dexamethasone) was combined with the cysteinyl leukotriene receptor 1 inhibitor, montelukast, to study their effectiveness on established asthma features in a murine model (8). Corticosteroid administration in conjunction with EGFR inhibition or surfactant administration would be of prime interest.

5.2 **Future directions**

Our studies have prepared the ground for future projects exploring several questions that have resulted from our findings. One such question is exploring the mechanisms by which surfactant inhibits mast cell mediators in the EAR. Our studies of calcium signals in mast cells triggered by allergen indicate that the manner of inhibition of the mast cells is via an indirect mechanism. Also given that the surfactant we used in our studies did not contain SP-A and SP-D, further exploring the potential inhibitory actions of surfactant phospholipids, or surfactant proteins B and C contained in the "natural surfactant" we used may provide some insight into the particular component of surfactant that reduces the EAR and by what mechanism it does so. If it is not the surfactant lipids at play, and if the roles of either SP-B or SP-C are demonstrated to be immunomodulatory, such findings would set a precedent in the present-day knowledge of the functions of surfactant proteins. SP-B and SP-C are more commonly associated with lung homeostasis but there have been reports that have implicated either SP-B alone or even a commercially-available surfactant, lacking SP-A and SP-D, in inhibiting inflammatory mediators releases from LPS or pathogen-induced macrophages (20; 28).

Furthermore, given that we demonstrated that surfactant could inhibit mast cells mediators, we were able to specifically show the inhibition of amphiregulin by surfactant, a ligand for the EGFR. Studying whether there is any EGFR expression on mast cells and what, if any, its potential role may be in the EAR though it may be too short a time for its activation.

We studied the gene expression of mediators released from the epithelium as the epithelium itself is important in airway remodeling and it potentially can influence ASM remodeling and phenotypic changes. In addition to epithelial-derived mediators we have already explored (TGFβ, MMP-9) as well as the neutrophilic chemotactic factor, Gro-α, the role of other epithelial-derived chemokines in ASM phenotype may be useful to explore. Recently, using human

ASM cells, it was reported that in a concentration-dependent manner, chemokines including eotaxin, RANTES, IL-8, and MIP-1α (macrophage inflammatory protein 1-α) increased ASM proliferation and decreased the rate of apoptosis (6).

Takeyama et al. (2008) reported that AG1478 (EGFR inhibitor) induced apoptotic goblet cells when administered after established goblet cell hyperplasia in a BN rat model of asthma (27). They concluded that EGFR played a role in maintaining goblet cells (27). It would be worthwhile to confirm the role of EGFR in goblet cell maintenance as observed in the study by Takeyama et al (2008) when AG1478 is administered concurrently to the allergen challenges as in the BN rat model of allergic asthma we have used for our studies, and not after established goblet cell hyperplasia. Along the same lines, studying whether inhibiting EGFR induces apoptosis of ASM in our BN rat model would provide more insight into the processes involved in maintaining ASM mass.

One of the advantages of our present methodology is the unique ability to capture a homogenous cell population using laser capture microdissection. We have been interested in ASM and epithelial cells in our studies. The subsequent RT-PCR results are specific to the chosen cell type. Thus continuing our study in chapter 4 where we have studied the ASM phenotype and how it relates to AHR and ASM remodeling 48 h after multiple allergen challenges in the sensitized BN rat, investigating how ASM phenotype evolves from the 48 h after the last allergen challenge of three challenges and compares to the gene expression at the 1 week time point is of primary interest.

Another point of potential interest is investigating whether corticosteroids are at all effective in modulating ASM phenotype. While there are discrepant data in clinical studies as to the benefits of corticosteroids in inhibiting airway remodeling, there are studies, including the one covered in chapter 3 in this thesis, that have reported that corticosteroids are effective in inhibiting airway

remodeling including inhibiting the increase of ASM mass after multiple allergen challenges (19; 25). An article by Henderson et al. (2006) reported inhibition of established goblet cells but not ASM by the corticosteroid dexamethasone given alone in an Ova-induced murine asthma model (8).

MicroRNAs (miRNAs) are 21- to 23-nucleotide noncoding RNA molecules that have been classified as important post-transcriptional regulators of cell growth, differentiation, and apoptosis (10; 34). Studying the role of miRNAs (miR) in regulating ASM phenotype in an *in vivo* model of asthma and to study how the data relate to the presence of AHR is of substantial interest to many investigative groups. miR-25, for one, has been reported to be involved in modulating ASM phenotype by influencing the expression of inflammatory mediators such as RANTES, eotaxin, and TNF-α, genes involved in extracellular matrix turnover, and contractile proteins, prominently myosin heavy chain (16). According to the authors, miRNA binding algorithms predict that miR-25 targets Krüppel-like factor 4 (Klf4), which is a potent inhibitor of smooth muscle-specific gene expression and also mediator of inflammation (16). They report that the inhibition of miR-25 in cytokine-stimulated ASM cells up-regulates Klf4 expression via a post-transcriptional mechanism (16). This provides novel evidence that miR-25 targets Klf4 in ASM cells and proposes that miR-25 may be an important mediator of ASM phenotype (16). miRNA 143 and 145 have also been implicated in regulating the proliferation and differentiation of vascular smooth muscle, targeting a network of transcription factors including Klf4, myocardin and Elk-1(3). Interestingly, miRNA expression has been reported to not be involved in the development of a mild asthmatic phenotype as studied in human biopsies or in the anti-inflammatory action of the corticosteroid budesonide (35).

It may be worth further exploring why and by what mechanism we observe an inhibition of inflammation, specifically eosinophils, when inhibiting EGFR in an *in vivo* asthma model. With our data, we ruled out the possibility of

it happening via epithelial-derived chemotactic mediators, eotaxin and RANTES. ASM-derived eotaxin and RANTES may be possible contributors however. Our MSD data also indicate no significant differences in IL-5 expression amongst the groups. Lipid mediators are also an important consideration. There are *in vivo studies* that have implicated the 5-lipoxygenase product, 5-oxo-6,8, 11,14-eicosatetraenoic acid (5-oxo-ETE) and leukotriene B_4 (LTB_4) as key chemotactic mediators that are responsible to recruit eosinophils (2; 26). Furthermore, stem cell factor (SCF) following antigen challenge may lead to the activation of and/or priming of mast cells for IgE-mediated release of inflammatory mediators, especially LTB_4 (13). There is also evidence of synergistic effects of various cytokines, chemokines, and adhesion molecules (15) that may influence eosinophilic recruitment, for example eotaxin with SCF-mediated LTB_4 (14).

Statement of Originality

In chapter 2, we reported the comparison of the assessment of large and small airway narrowing using the constant phase model and a morphometric analysis performed on frozen lungs. For the first time, the constant phase model was used to assess mechanical parameters of the EAR. We further assessed whether exogenous, natural surfactant, not containing the conventional immunomodulatory surfactant proteins, has ameliorating effects on EAR. Unexpectedly, the collectin-free surfactant of choice was effective in inhibiting bronchoconstriction via immunomodulatory effects. Collectively, these are novel findings and further investigation is required into the downstream signaling of the component(s) of surfactant responsible for the inhibition of airway narrowing.

In chapters 3 and 4, the relationship between AHR and airway remodeling, specifically ASM growth and/or ASM phenotype, was explored in an *in vivo* asthma model. It is safe to conclude that they are not linked, as at 1 week after allergen challenges when AHR begins to dissipate airway remodeling persists.

The constant phase model was used to assess airway function and the site of airway responses in a rat model of asthma and its comparison with the partitioning of airway remodeling into central/peripheral airways was implemented for the first time in our studies. Budesonide, a corticosteroid was used as a probe in one of these studies with the basis it may highlight differences between AHR and ASM, given that it is an anti-inflammatory agent. Using a corticosteroid as a probe was an additional insightful step.

In chapter 4, EGFR inhibition was also assessed in both AHR and remodeling but again, with the partitioning of the responses for both asthmatic features. ASM phenotype was assessed with the aid of laser capture microdissection to capture a homogenous population of cells. One group of investigators (36) has used this technique to assess modulation of ASM phenotype but none have used this technique in an animal model to assess ASM phenotype. We have confirmed the feasibility of its application to the rat and to use it to study the transcriptional regulation of muscle proteins after allergen challenge. We are the first to assess the potential effects of EGFR inhibition on

ASM phenotype which has not been reported as of yet in an *in vivo* model of asthma or otherwise.

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Research Compliance Certificates