AIRWAY REMODELING

AIRWAY REMODELING IN MOUSE MODELS OF EXPOSURE TO ALLERGEN

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ABSTRACT

Asthma is a respiratory disease that affects over 300 million people world-wide and is involved in over 250000 deaths annually. Asthma is classically thought of as an allergic disease with variable airflow obstruction and airway hyperresponsiveness (AHR) associated with airway remodeling, although phenotype variations are observed in the population. Due to multiple factors including genes, gender, exposure to pathogens, environmental pollutants, diets, and obesity, a clear picture of the complex interactions between an individual's genes, the environment which they live in, and the development of an asthmatic phenotype remains elusive. Despite the availability of treatment strategies for asthma, varying degrees of airway inflammation, remodeling, and AHR remain present in an asthmatic patient.

Our general hypothesis that was the basis for all studies was the following: "Airway remodeling in response to allergen exposure is a major contributing factor to AHR observed in asthmatics. Understanding the mechanisms behind the different components of airway remodeling will provide new avenues for therapeutic development aimed at improving lung function above and beyond current treatment strategies."

The currently available therapeutics and management strategies for asthma are unable to prevent or reverse components of airway remodeling. It is possible that with greater understanding of the processes involved in the various indices of airway remodeling new classes of therapeutics could be developed to selectively target this broad, ill-managed aspect of asthma. Collectively the studies contained in this thesis have been linked by the general themes of greater characterization of *in vivo* mouse

models of allergen exposure, the application of these models to mechanistically explore the biological pathways involved in the different components of airway remodeling, and the testing of novel therapeutic strategies targeting these pathways of interest.

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My thesis was not distinct from my life, so all those that I have met along the way deserve some acknowledgement. Unfortunately, I have met so many inspiring people that it would take up too much space.

Here's to you all – I raise a glass – and in the immortal words of Trooper,

"We're here for a good time, not a long time, so have a good time, the sun can't shine everyday".

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CHAPTER 1:BACKGROUND

Introduction

The central focus of this thesis is on mouse models of experimental asthma with the major aim being to observe and explore mechanisms involved in changes to the airway wall structure. The results of the studies contained within this thesis have been interpreted in a manner whereby the potential relevance to humans was addressed. The thesis is being presented in a "sandwich" format where submitted/published publications exist as the meat and introduction/conclusions serve as the bread.

To provide proper context and highlight the clinical relevance of the thesis, an introduction of sufficient depth must be provided to the reader. Of particular importance to discuss is the worldwide burden of asthma and the factors that may be contributing to this disease process. Seminal clinical studies and methods that have helped define asthma as an inflammatory disease with changes in airway function must be discussed to give a foundation for the experimental animal modeling systems used throughout this thesis. We follow with discussion of some of the pathological changes observed in human asthmatics and how we can appropriately model and analyze these changes in animal model systems, specifically mice. A general hypothesis and theme for the thesis are stated before concluding the introduction with a summary of the findings and the relationship between the studies contained within the thesis.

Worldwide Asthma Burden

Asthma is a respiratory disease that affects over 300 million people world-wide(1). The prevalence of asthma is highest in developed nations at approximately 20%,

and is rising from lows of 1 % in developing nations(1). The reasons for the increase in prevalence of asthma are unknown. The World Health Organization estimates that 250000 people die each year due to asthma(1). The considerable social and economic costs associated with asthma include absence from school, hospital visits, patient admission, lost productivity in the work place, and death. The rise in asthma worldwide is observed in both children and adults and is positively associated with the adoption of a Western lifestyle. For this reason, it is anticipated that as populations in countries such as Brazil, Russia, India, and China adopt a Western lifestyle the prevalence of asthma will increase significantly. The burden in both developed and developing countries exists despite the availability of effective management strategies for asthma(1). Unfortunately it appears that the available strategies are unable to prevent the development of or to eliminate existing asthma, but instead are best suited for management of the disease(2). Clearly there is need for alternate strategies to improve patient care and disease management aimed at reducing the prevalence of asthma and reversing an existing condition.

Brief Summary of Asthma

Asthma is most often characterized by airway hyperresponsiveness (AHR), inflammation, and remodeling(3-5), although heterogeneities of phenotypes exist within the patient population(6). AHR refers to the increased tendency for airways to narrow in response to a bronchoconstrictor (e.g. histamine) resulting in airflow obstruction. In the context of asthma, the often observed airway inflammation is an aberrant response of the

immune system resulting in specific cell recruitment to combat an inhaled innocuous substance(s) called an allergen(3). Cellular inflammation is commonly associated with increased circulating immunoglobulin E (IgE) levels and development of allergy to various allergens. Repeated allergen inhalation leads to chronic airway inflammation that is also associated with AHR. Chronic inflammation is thought to play a role in initiating and perpetuating airway remodeling, a term that describes structural changes to multiple components of the airway wall(4,5,7). Considering this brief summary, asthma is classically thought of as an allergic disease with variable airflow obstruction associated with airway remodeling, although variations exist within the population(6).

Factors Influencing the Development of Asthma

Multiple factors including genes, gender, exposure to pathogens, environmental pollutants, diets, and obesity, all have evidence to support their influence in the development of asthma(1). Genetic analysis in family studies(8,9), segregation analysis(10), and linkage studies(11) reveals a strong genetic component to AHR and development of elevated IgE levels (8,12). The 5q chromosome region contains many molecules associated with the asthma phenotype including, interleukin-4 (IL-4), IL-5, IL-13, and granulocyte macrophage colony stimulating factor(8,10). The gender of an individual may also be an important risk factor in asthma(13). A prospective study examining a broad cohort of individuals from age 9 through to age 26 in New Zealand demonstrated that female sex is a risk factor for asthma(13). From this work it appears possible that changes in expression of female sex hormones (estrogen, progesterone)

during puberty may be associated with the development of asthma(13). Although substantial evidence exists to support a genetic and sex component to asthma, the environment, and the temporality of these exposures, appear equally important(14). A population study examining the development of asthma and allergy in children demonstrated that exposure to older siblings and other children at home or in day-care settings in the first 6 months provides protection from development of asthma in later years(15). This study did not systematically examine the environment for allergens/pathogens but supports the notion that genes do not alone determine the presentation of asthma. A more systematic approach examining the development of allergy in relationship to environment demonstrated that house dust mite (HDM) and cat allergen are unique in their ability to sensitize individuals(16). Whereas increasing concentrations of cat allergen in the home environment lead to a tolerant phenotype, this was not observed for HDM(16). The demonstration that different allergens elicit different immune responses adds complexity to the role that our environment plays in development of allergy and asthma(14). An example of this complexity can be observed between two separate studies examining exposure to rural environments/substances(17,18). In a Dutch population, rural environment exposure appears to be protective against developing allergy and asthma symptoms(18). In contrast, a similar population in New Zealand showed an increase in allergy associated with rural environment exposure(17). These confounding results may be due to differences in allergen/pathogen exposure levels, timing of exposure, genetic makeup of the population, differences in diet(19), or obesity(20). Due to these multiple factors, a

clear picture of the complex interactions between an individual's genes, the environment which they live in, and the development of an asthmatic phenotype remains elusive.

Clinical Assessment of AHR

AHR is a term that encompasses changes in airway sensitivity, reactivity, and maximum airway narrowing, all of which are observed in asthmatics. Increased airway sensitivity refers to the tendency of the airways to contract to concentrations of bronchoconstrictors that would normally not cause airway narrowing. Increased airway reactivity is an index of the degree of change in airway tone for a given increase in the concentration of the agonist. An asthmatic will also possess increased maximum airway narrowing as compared to non-asthmatics. The exact cause(s) of each of these components of AHR are currently unknown.

To assess for AHR in humans, airway function measurements are made in the clinic by observing the ability of the airways to narrow to an inhaled bronchoconstrictor agent such as methacholine or histamine(21,22) with the use of spirometry. A spirometer is used to measure lung volumes and flows inhaled and exhaled voluntarily by a subject and provides insight into lung function(23). In this scenario, a subject under clinical monitoring is required to inhale nebulized bronchconstrictor for a fixed duration. Following the nebulization, the subject is required to expire air into a spirometer using a maneuver called a forced expiratory volume in 1 second (FEV_1). The FEV_1 is used clinically as a measure of airway function(21,22). The concentrations of inhaled bronchoconstrictor are increased until the subject records a 20% drop in their FEV_1 –

indicative of a reduced ability to exhale air as a result of changes in lung function. The concentration of bronchoconstrictor that results in the 20% drop is called the provocative concentration 20 (PC_{20}) and is a measure of airway responsiveness. The inhalation of bronchoconstrictor is stopped once the PC_{20} value is obtained to prevent excessive airway narrowing that would occur in asthmatics with higher concentrations. Complete dose response curves are not obtained in the clinic as PC_{20} values, rather than plateaus in bronchoconstriction, are the outcome measured in clinical analysis. This is in contrast to animal studies of asthma (see below : Methods of determining AHR and Airway Remodeling in Mice). The PC_{20} values can be used to characterize the degree of an asthmatics airway dysfunction as severe, moderate, or mild, with the cut-off of a non-asthmatic being a PC_{20} value greater than a bronchoconstrictor concentration of 16mg/ml (Figure 1) (22,24). The range of PC_{20} values from severe asthmatics to non-asthmatics may vary by a factor of up to 10000. The mechanisms underlying the drastic differences in PC_{20} values observed between asthmatics and non-asthmatics are unknown.

Early investigational studies attempting to determine the etiology of AHR observed in asthma employed allergen inhalation challenges and monitored subsequent airway physiology(25). Optimized methods of allergen inhalation challenges subsequently demonstrated the role of IgE and observed an increase in eosinophils associated with AHR(25). With the foundations laid for implication of inflammation in AHR, the characterization of inflammatory cell profiles, cytokines, chemokines, receptors, proteases, immunoglobulins, and the complex interplay between these players has been explored in clinical, *in vivo*, *ex vivo,* and *in vitro* experimental systems (see

below – Modeling Asthma). Despite these studies, the precise contribution of the various inflammatory players to AHR remains elusive.

Inflammation in Asthma – Classical Model of Allergy Development

Cockcroft and colleagues' seminal work linking an inflammatory component to AHR provided a firm foundation for further research in clinical subjects. To detect inflammation in the airways of asthmatics various invasive biopsy (26) and noninvasive (27) methods were developed and validated in the clinic. These procedures helped elucidate both the cellular and fluid-phase mediators associated with the phenotype variations of the asthmatic population. The inflammatory cell types commonly associated with the asthma phenotype include eosinophils, mast cells, CD4+ T cells, basophils, and macrophages. As outlined above, heterogeneity exists in the population of asthmatics as evidenced by varying inflammatory profiles(6). It is now becoming apparent that structural cells are capable of generating/perpetuating an inflammatory response, including epithelium(28), fibroblasts(29), and ASM cells(30). A detailed review of each cell type and the data discussing their role in asthma is beyond the scope of this thesis but has been reviewed elsewhere (28-30).

The classical initiation of an allergic phenotype requires that antigen presenting cells (e.g. dendritic cells, macrophages, B cells) internalize and process foreign antigen resulting in presentation of antigen via MHC class II molecules on the cell surface. The foreign antigen is described as an allergen once an adaptive immune response is developed against the given molecule/substance. Examples of antigens that become

common allergens are a house dust mite protein, Der p1, and a cat protein, Fel d1. Activated antigen presenting cells with antigen peptide fragments presented on their cell surface then migrate to nearby downstream lymph nodes. It is in these lymph nodes that circulating blood flow containing T cells with their varied receptors can recognize foreign peptide in the context of MHC presenting molecules on the surface of antigen presenting cells. In allergy, antigens presented in the context of MHC class II activate CD4+ T cells via an interaction between MHC class II/peptide fragment/CD4/TcR. The development of Th1 or Th2 CD4+ T cells depends on the type of antigen being presented but also the current cytokine environment. Antigen presenting cells in lymph nodes presenting foreign molecules associated with allergy typically produce activated Th2 CD4+ T cells, which then proliferate in the lymph nodes and begin to migrate to the original site of exposure. Within the lymph node follicles the activated CD4+ T cells may activate B cells to begin producing and secreting IgE that recognize the same antigen. Secreted and circulating antigen specific IgE is able to bind to any cells expressing the high affinity FCeRI, including mast cells, eosinophils, and basophils. The recruitment of activated CD4+ T cells to the original site of exposure is accompanied by a production of Th2 cytokines including IL-4, IL-5, and IL-13 and chemokines eotaxin 1. The secretion of various cytokines/chemokines by activated CD4+ T cells at the site of original exposure results in recruitment and proliferation of more inflammatory cells from the bone marrow. Subsequent exposure to antigen may result in IgE binding of ligand and cross-linking of the FCeRI receptors on the surface of eosinophils, mast cells, and basophils. Cross linking of the FCeRI receptors by antigen binding to IgE results in the activation of

these inflammatory cells and release of granules of pre-formed mediators and generation of lipid mediators that further the inflammatory response. Both infiltrating and resident inflammatory cells are capable of liberating reactive oxygen species and eicosanoids such as leukotrienes that can act directly on ASM to induce potent contraction of airways. It is postulated that repeated exposure to antigens results in chronic airway inflammation that influences the phenotype of structural cells including airway epithelium, airway smooth muscle, and fibroblasts. The bridge between the chronic inflammation and changes in the architecture of the airways has not been causally linked in clinical studies, although substantial data exists from animal models associating the relationship (see below : Small animal models of allergen exposure).

Airway Remodeling in Asthma

Changes in structural cell biology and associated inflammation are thought to contribute to the many different changes in airway structure collectively called airway remodeling(3,4,7). The broad, all encompassing term airway remodeling, may include changes in airway epithelium integrity(31), goblet cell metaplasia(32), increased ASM content(33,34), increased matrix deposition(2,35), angiogenesis beneath airway basement membranes(36) and altered neural factors(37). The biological processes behind each structural change may or may not involve common mediators/pathways. It follows that studies presented in this thesis are designed to explore the individual components that contribute to general process of airway remodeling.

Before discussion of airway remodeling can be performed, we must first consider the state of the non-asthmatic airway wall. The first structural line of defense in the lung is comprised of the ciliated bronchial epithelium that forms a barrier with both mechanical and physical properties. Ciliated epithelial cells, basal cells, and Clara cells all work together in harmony to produce controlled levels of mucus, contain inhaled foreign antigens, and propel them to the proximal airways where they are cleared by cough or swallow(38,39). In addition to the viscous mucus layer, which protects the epithelium, between epithelial cells a physical barrier is created by formation of tight junctions at the apical and basal levels between adjacent cells. These junctions consist of interactions between E-cadherin, claudins, occludins, and ZO-1(39). The tight junctions, mucus secretion, and mucociliary actions of the airway epithelium form a crucial protective barrier preventing unwanted foreign antigens from invading deeper into the lung tissue.

The epithelium is associated with the basement membrane, a foundation consisting of the lamina propria and the lamina reticularis. The lamina propria is secreted by the epithelium and acts as the primary anchor. It is essential for determining the polarity of epithelial cells and facilitating repair of the epithelium by promoting adhesion and migration(40) The lamina reticularis consists of matrix proteins including collagen I, III, and fibronectin and is primarily synthesized by fibroblasts (35,40)

Whereas the epithelium and basement membrane clearly have mechanical and physical properties required for preventing antigen from invading the lung tissue, the role of ASM is less clear(41,42). Residing beneath the basement membrane, ASM is capable

of narrowing the airway lumen in response to stimulation by contractile agonists released from neurons, inflammatory cells, and structural cells. The classical method of ASM contraction requires a stimulus that results in an increase in intracellular calcium concentration, which is usually released from internal stores. In a ligand based scenario, a contractile agonist such as acetylcholine binds to its receptor on the surface of the ASM cell resulting in activation of the GPCR. G protein activation results in activation of cytosolic enzymes capable of continuing and amplifying the ligand signal. G protein activation of lipid metabolizing enzymes results in the liberation of inositol triphosphate from PIP_2 at the surface of plasma membrane. IP_3 is subsequently capable of releasing sequestered intracellular calcium stores in sarcoplasmic reticulum by binding to the IP_3 receptor. Elevations in intracellular calcium levels are required for calmodulin and subsequent MLCK activation, ultimately leading to myosin light chain phosphorylation. Myosin light chain phosphorylation results in activation and a significant change in the confirmation of the actin/myosin heavy chain interactions allowing for the ATPase activity in the myosin head to fuel contraction. The actin and myosin filaments that form crucial parts of the contractile machinery are anchored to the cell membrane and dense bodies that contain an intermediate filament called desmin. The contraction of ASM results in airway narrowing and changes in lung function. Although mechanisms responsible for ASM contraction are understood in depth, great debate exists as to the evolutionary relevance of ASM in the lung and whether or not it provides any significant physiological benefit (41,42).

As described above, the process of airway remodeling involves abnormal and pronounced changes to the various structures that make up the airway wall. The bronchial epithelium has been reported to be more fragile(43), contain regions where epithelium has been denuded(31), and overall possesses reduced physical barrier properties(40), perhaps due in part to allergen induced cleavage of tight junctions(44,45). The epithelium also undergoes changes to non-ciliated mucus producing cells in a process called goblet cell metaplasia(46,47). Cilia beat frequency may also be reduced in asthmatic environments suggesting impairment in the ability to remove inhaled particulates and mucus(48). Mucins, a major content of goblet cells, are heavily glycosylated molecules defined by tandem repeats of amino acids(49). These molecules can be secreted via vesicles or released on the cell surface in tethered forms. Stimulation of mucus secretion is followed be release into the airways, where mucins may mix with water, proteins, ions, DNA, and/or albumin, depending on the airway environment(50). The major mucin molecule produced by goblet cells is MUC5AC, while other mucins such as MUC2 and MUC5B are less abundant(49). The molecular processes that direct goblet cell metaplasia are not completely understood, although both inflammatory mediators(51,52) and growth factors(47,53) have been implicated using *in vitro* and *in vivo* models. Strategies aimed at preventing production or secretion of excessive mucus complemented with mucolytic agents may prove useful in the future. Important to emphasize, the functional consequences of goblet cell metaplasia are severe. Many fatal cases of asthma are accompanied by mucus plugging in the airways from 2nd generation

to terminal bronchioles(54). **Greater understanding of the mechanisms responsible for goblet cell metaplaisa is of relevance to asthma management.**

The basement membrane provides a physical foundation for the epithelium to anchor to and can undergo thickening in asthmatics, contributing to the airway remodeling process. The lamina reticularis component of the basement membrane normally consists of reticulin but undergoes significant increases in content of matrix as well as changes in matrix expression(35). Increases in the content of collagen I, III, and fibronectin are observed(35). The increased matrix content is capable of sequestering growth factors(55), becoming hydrated with edema(56), and influencing the phenotype of neighboring structural cells(57). The functional consequences of a thickened basement membrane are not clear and have been debated in pro-con editorials in leading respiratory journals(58). Some studies have reported an association between basement membrane thickness and decrease in lung function, while others show no association (2,31,50,59). The arguments for the contribution of basement membrane thickening to airway dysfunction include exaggerated airway narrowing due to changes in airway wall mechanics, changing of phenotype of underlying ASM, and altered tethering of airway wall to parenchyma. In contrast, it is possible that the increased thickness of the basement membrane may act as an increased load that ASM must overcome to reduce airway caliber. It is possible that the generation of the airway tree that basement membrane thickening occurs is of functional importance, but this has not been addressed in the clinic(60). Future application of clinical imaging techniques paired with lung function tests may provide insight into the functional consequences of basement

membrane thickening. **The role of basement membrane remodeling and the functional consequences remain to be determined.**

The ASM is the primary modulator of airway caliber and undergoes changes in the airways of asthmatics. Classical studies examining the airways of asthmatics vs. non-asthmatics revealed both an increase in the size (hypertrophy) (33) and number (hyperplasia) (34) of ASM cells in asthmatics. Recent data has suggested that ASM cells can also migrate and this may play an important role in the remodeling process(61). The *in vitro* culture system has been integral in exploring the mechanisms responsible for ASM growth and migration and have demonstrated the varying roles of PDGF-BB, FGF-2, TGF-β1, IL-4, and IL-13 in these processes(62-65). The consequences of hyperplasia//hypertrophy/migration ultimately are an increase in the mass of ASM. Mathematical modeling of increased ASM mass has demonstrated that this tissue could be a key player in excessive reductions in airway diameter observed in asthmatics (66,67). These modeling studies did not investigate the possibility that the ASM could undergo phenotypic changes that could alter function(68,69) something that has been addressed in using *in vitro*, but not *in vivo* studies. To date no pharmacological intervention exists to selectively ablate increases in ASM mass, although an invasive procedure has been developed and undergoing regulatory approval(70,71). **The benefit of the procedure outlines the importance of selectively targeting ASM in asthmatics.**

Therapeutics in Asthma

The therapeutics available for asthma are broadly classified as either controllers or relievers(1). Disease controllers consist of therapeutics with anti-inflammatory properties or are taken on a long-term steady basis. This class of compounds includes glucocorticoids, leukotriene modifiers, long acting $beta_2$ adrenergic agonists (when combined with glucocorticoids), and anti-IgE therapies. Reliever therapies are most often rapidly acting $beta_2$ adrenergic agonists. The Global Initiatives for Asthma has outlined the use of these therapeutics and suggests control of disease is a primary goal of a physician(1).

It is important to emphasize that despite ongoing treatment and control with both classes of therapeutics, AHR still persists in asthmatic patients as assessed by PC_{20} values(2). An asthmatic individual may observe a 1-2 doubling concentration improvement in their PC_{20} value – a measure of AHR (see above), while on appropriate doses of corticosteroids(2). Although of clinical importance, this improvement does not normalize asthmatic lung function to that observed in non-asthmatics. This demonstrates that there is a sustained component of AHR that is not effectively treated by current therapeutics. We postulate that airway remodeling components contribute to this sustained AHR(72). Reports from both *in vivo* models and clinical studies demonstrate that current clinically relevant therapeutic strategies are not capable of normalizing/reversing established airway remodeling indices. **The development of new pharmacological tools able to selectively target goblet cell metaplasia, basement**

membrane thickening, or ASM hyperplasia/hypertrophy will help explore the broad hypothesis that components of airway remodeling contribute to AHR.

Modeling in Asthma Research

Clearly the development of new therapeutc agents will require the complementary use of various experimental modeling systems. A central feature to medical science research is the use of model systems, with the ultimate goal of translating promising results to the clinic. For this reason model development should be performed with attention to how obtained results are relevant to the clinical condition. Modeling of any disease or biological process can include *in vitro, in vivo, ex vivo*, and *in silico* – each with their own strengths and weaknesses. Asthma research employs a wide variety of *in vitro* culture models that explore the biology of ASM(68), airway epithelial cells(40), fibroblasts(29), inflammatory cells from lung and/or circulation. Animal models of asthma are another tool in the researcher's kit that can be used to explore the *in vivo* interactions between various cells types. *In vivo* models can include the use of sheep, horse, dog, cat, although more routinely focus on rat and mouse(73). *Ex vivo* models are hybrids of *in vitro* and *in vivo* models and involve the removal of a tissue/organ(s) of interest from an animal or patient and investigating the biology of the material outside of the body. Functional responses of ASM strips and airways in precision lung slices are two *ex vivo* methods commonly used in asthma research(74-76). *In silico* or computer modeling studies provide another opportunity for researchers to explore possibilities that may be limited or impossible with other model systems(66,67). Mathematical modeling

of the functional consequences of selectively changing various airway wall components gives researchers insight that could not be obtained from *in vitro* or *in vivo* models. The important point to emphasize and always consider when using models or interpreting data obtained from models is their strengths and limitations. By definition they are models and therefore are only an approximation of the given clinical biological system being studied.

Models of Allergen Exposure to Small Animals

Our group models asthma by exposing mice to an allergen and observing the inflammatory, airway remodeling, and functional responses. We perform these studies by exposing mice to allergen for varying durations. As mice do not intrinsically develop asthma, the development of an allergic phenotype to a given antigen must be promoted artificially. Exposure to chicken egg ovalbumin (OVA) (77) has been used as a model of allergic sensitization in a variety of incarnations and was adapted to models of airway disease in the early 70s (78). Used widely by investigators, the common model of OVA sensitization requires injection of OVA conjugated to an adjuvant to stimulate an immune response towards what would normally be an innocuous protein. Subsequent exposure to the airways elicits inflammatory and functional responses similar to those observed in humans. Historically the Inman laboratory has used an OVA model of allergen sensitization and exposure(60,79-81). Although a commonly used model, we do not feel that this is the most relevant model available as the requirement of an adjuvant highlights the differences between this system and naturally occurring allergy and asthma. Pursuits

of adjuvant free allergen exposure models has lead to the development and application of house dust mite (HDM) (82), cockroach(83), and ragweed (84) models. We now employ both OVA and HDM models in our lab(85,86).

It is important to emphasize that there are varying degrees of freedom in model development that must be considered when designing a study and interpreting results (Figure 2). Small animal choices for models of allergen exposure routinely use mouse and rat, while guinea pig is less often used. Each species can be made available in a variety of strains in varying ages and sexes. Strains of both mouse and rat have been shown to vary in intrinsic airway responses to bronchoconstrictors and their inflammatory profiles during allergen exposure (87-90). The majority of animals are purchased through animal suppliers that have tightly controlled breeding colonies. We have identified further differences in investigator housing facilities (e.g. conventional vs. ultraclean HEPA filter) that promote differences in allergic responses in the airways. All of these variables must be considered even before the actual allergen model is implemented, as they *will* contribute to deviations in results. As mentioned, there are multiple allergens that can be used for exposure including the most common OVA, with more common use of *relevant* allergens including HDM(82), ragweed(84), and cockroach(83) extracts. Each allergen can be suspended in solution and adapted for intranasal, intratracheal, or aerosolized exposure, with each route having a distinct distribution profile. Southam and colleagues have shown that allergen delivery distribution via intranasal route is dependent on volume(86). The chosen length of exposure to the allergen will dictate the development of airway inflammation, airway

remodeling indices, and associated AHR. Exposure protocols are classically considered brief/acute or chronic. Brief allergen exposure protocols are useful in examining the immune mediated events, while chronic protocols are more capable in generating simultaneous development of several airway remodeling indices. A final variable that can be varied in small animal models is the amount of recovery time that is allowed following the final exposure period. It has been demonstrated with both OVA and HDM models that recovery periods following chronic allergen exposure induced inflammation allow resolution of these mediators to baseline levels, while still maintaining structural and functional changes (72,80). Despite the many possible permutations in allergen model design, recurrent themes of AHR, goblet cell metaplasia, basement membrane thickening, and increased ASM content following allergen exposure have been observed in guinea pigs (91), rats (92-96), and mice (72,80,85).

Methods for Determining AHR and Airway Remodeling in Mice

The methods for determined AHR in mice are different than in humans. There exist invasive and non-invasive methods for measurements of airway physiology in mice that have been discussed in detail elsewhere(97). Recently, editorials in leading respiratory research journals have declared the classical non-invasive whole body plethysmograph method to be an indirect and inaccurate measure of lung function in mice (98-100). Invasive methods require anesethesia, intubation of the trachea, and mechanical ventilation at user determined volumes/rates while data is collected on a computer system. Methacholine used as a bronchoconstrictor, can either be nebulized or

injected intravenously in increasing doses while lung function is monitored. Changes in airway pressure are monitored by the ventilator system and provide information on total respiratory system resistance and elastance. More complex modeling systems can be employed in attempts to delineate where in the lung (parenchyma vs. airway) the changes in mechanics are occurring, but are beyond the scope of this thesis.

Airway remodeling analysis in animal models uses histochemical and immunohistochemical techniques similar to clinical pathological analaysis. Basement membrane thickening can be identified with Masson's Trichrome, Picrosirius Red, or Hematoxylin and Eosin stains. Antibodies for specific basement membrane components such as fibronectin, laminin, collagen I, and collagen III, can also be used for analysis of basement membrane composition.

Goblet cell metaplasia analysis is routinely performed with an alcian blue/periodic acid Schiff histochemical stain, while complementary immunohistochemical stains for specific mucins are used less often.

Changes in ASM in small animal models of asthma are most commonly determined by performing α-smooth muscle actin (α-SMA) immunohistochemistry (IHC) on lung sections (80,85,93-95). Ultimately, α-SMA staining is only useful for determining the amount of area stained positive for this protein of interest and limited extrapolation to amount of ASM content should be made. Additionally, the use of α-SMA staining affords little insight if any into the hypertrophy/hyperplasia of smooth muscle. The use of the thymidine analog, bromodeoxyuridine (BrdU), has afforded investigators the ability to investigate smooth muscle cell growth by labelling cells as

they proliferate and continue through the S phase. Using monoclonal antibodies that are selective for BrdU (101), DNA synthesis associated with cell division can be monitored by counting the number of positively stained nuclei. In small animal models of allergen exposure BrdU is most commonly administered via intraperitoneal injection(94,95) around the periods of exposure, although oral routes through drinking water have also been validated (102). Using this approach investigators have been able to demonstrate allergen induced increases in ASM hyperplasia in rat models of OVA exposure (94,95). More recently the use of monoclonal antibodies for proliferating cell-associated nuclear antigen (PCNA) have been used to assess smooth muscle cell proliferation without the requirement for BrdU administration(103). The absence of a smooth muscle specific marker provides an opportunity for incorrect interpretation of conventional IHC. Although α-SMA IHC remains to be routinely used as a marker of ASM cells, it has been widely acknowledged that myofibroblasts will also stain positive for this marker. For this reason, to more confidently assess changes in ASM content with IHC, multiple stains for different smooth muscle markers (calponin, desmin, smooth muscle myosin heavy chain) should be performed(68).

Regardless of what airway remodeling component is being stained for, systematic, controlled, and blinded analysis must be performed. We have developed morphometric methods that allow for analysis of goblet cell metaplasia, basement membrane thickening, and changes in ASM mass (60,104). Morphometric techniques address the lack of quantitative analysis of stand alone histology.

General Hypothesis and Theme of Thesis

Our general hypothesis that was the basis for all studies was the following:

"Airway remodeling in response to allergen exposure is a major contributing factor to AHR observed in asthmatics. Understanding the mechanisms behind the different components of airway remodeling will provide new avenues for therapeutic development aimed at improving lung function above and beyond current treatment strategies."

Our general theme for this thesis was the following:

"Use *in vivo* mouse models of exposure to allergen for a) observing the consequences of allergen exposure, b) mechanistically determining biological pathways involved in airway remodeling, and c) developing and testing novel therapeutic strategies."

The available therapeutics and management strategies for asthma are unable to prevent or reverse components of airway remodeling. It is possible that with greater understanding of the processes involved in the various indices of airway remodeling new classes of therapeutics could be developed to selectively target this broad, ill-managed aspect of asthma. Indeed, recent non-pharmacologic selective intervention procedures aimed at reducing ASM content are substantiating the need to targeting components of airway remodeling(70,71). The development of medical procedures or pharmacological agents selectively targeting different indices of airway remodeling will ultimately require the use of models to test for efficacy of treatment. Characterization of models with concern for outcome measures relevant to the process being studied is essential. In our first study, Chapter 2 - REGIONAL DIFFERENCES IN THE PATTERN OF AIRWAY REMODELING FOLLOWING CHRONIC ALLERGEN EXPOSURE IN MICE, we

observe and report the heterogeneous nature of basement membrane thickening in airways of mice following chronic exposure to OVA. By characterizing the remodeling patterns in our model we provide evidence that suggests basement membrane thickening in the clinic may not be a homogeneous process throughout the entire airway tree. This may be true for other components of airway remodeling as well. Our second study , Chapter 3 - THE ROLE OF STAT6 AND SMAD2 IN A MODEL OF CHRONIC ALLERGEN EXPOSURE : A MOUSE STRAIN COMPARISON STUDY, further characterized our model of chronic OVA exposure and mechanistically linked IL-13 to inflammatory patterns observed only in mice that demonstrated increases in airway remodeling indices. Greater characterization and mechanistic analysis of our OVA model provided insight into the molecular pathways that may be involved in promoting increases in airway remodeling indices and highlighted potential new therapeutic targets(105). The third study contained in this thesis, Chapter 4 - AN ESSENTIAL ROLE FOR A NOVEL EPITHELIAL GABAERGIC SYSTEM IN MUCUS OVERPRODUCTION IN ASTHMA, demonstrates the use of animal models of exposure to allergen to test novel hypotheses related to the goblet cell component of airway remodeling. **Although my involvement was limited in this study,** it relates to theme of the thesis of using animal models to mechanistically investigate pathways involved in airway remodeling and was followed up in later studies where I was the primary investigator (Chapter 5). This study provided evidence to support an IL-13 dependent GABAergic signalling mechanism in the airway epithelium that resulted in goblet cell metaplasia using various strategies including OVA exposure mouse models. The

mechanisms behind the IL-13 dependent GABAergic system in airway epithelium were further explored in Chapter 5 - THE ROLE OF IL-4Rα IN THE INDUCTION OF GLUTAMIC ACID DECARBOXYLASE IN AIRWAY EPITHELIUM FOLLOWING BRIEF HOUSE DUST MITE EXPOSURE. Applying our HDM exposure model, we observe similar changes in GABAergic signaling as compared to our OVA model. We also mechanistically link the IL-4Rα to the IL-13 mediated changes in epithelium GABAergic activity using a monoclonal antibody class of therapeutic. Our data showing the efficacy of an IL-4Rα monoclonal antibody support further research and development of these classes of compounds for use in the clinic. This study also substantiated our previous findings that IL-13 is playing a central role in the in the airway remodeling process as evidenced in Chapter 3 and supported by the literature (51,52). Characterization and mechanistic studies were also performed to greater understand the ASM component of airway remodeling. In Chapter 6 – THE ROLE OF PLATELET DERIVED GROWTH FACTOR-BB IN AIRWAY SMOOTH MUSCLE PROLIFERATION IN MOUSE LUNG, we further characterize our OVA model demonstrating that ASM cell hyperplasia is the dominant mechanism of increased muscle mass. We also mechanistically explore the hypothesis that platelet derived growth factor-BB is a potent ASM mitogen responsible for hyperplasia observed in airway remodeling.

Collectively the studies contained in this thesis have been linked by the general themes of greater characterization of *in vivo* mouse models of allergen exposure, the application of these models to mechanistically explore the biological pathways involved

in the different components of airway remodeling, and the testing of novel therapeutic strategies targeting these pathways of interest.

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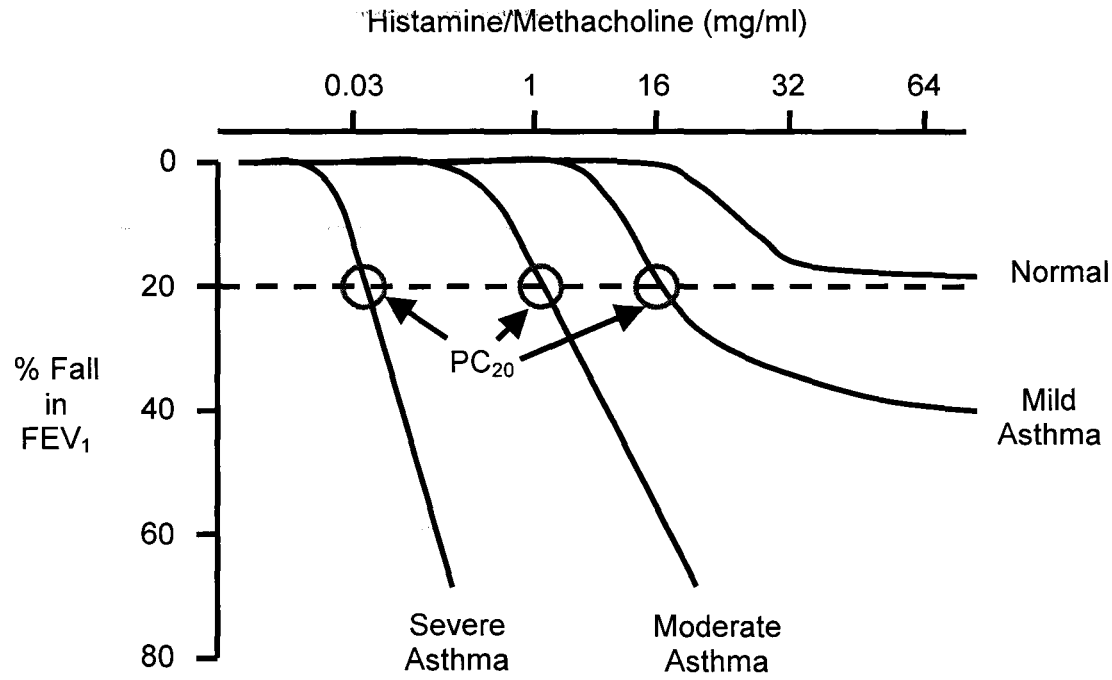

Figure 1 : Airway responsiveness of asthmatics and normal subjects. Airway hyperresponsiveness is defined as a PC_{20} value less than 16mg/ml (mild asthma). Lower PC_{20} values of 1mg/ml (moderate asthma) and 0.03mg/ml (severe asthma) are observed in the population. Normal subjects may not achieve a 20% fall in FEV_1 even at high concentrations of histamine/methacholine (>64mg/ml). Drops in FEV_1 greater than 20% are minimized in the clinic to prevent taking unnecessary patient risks.

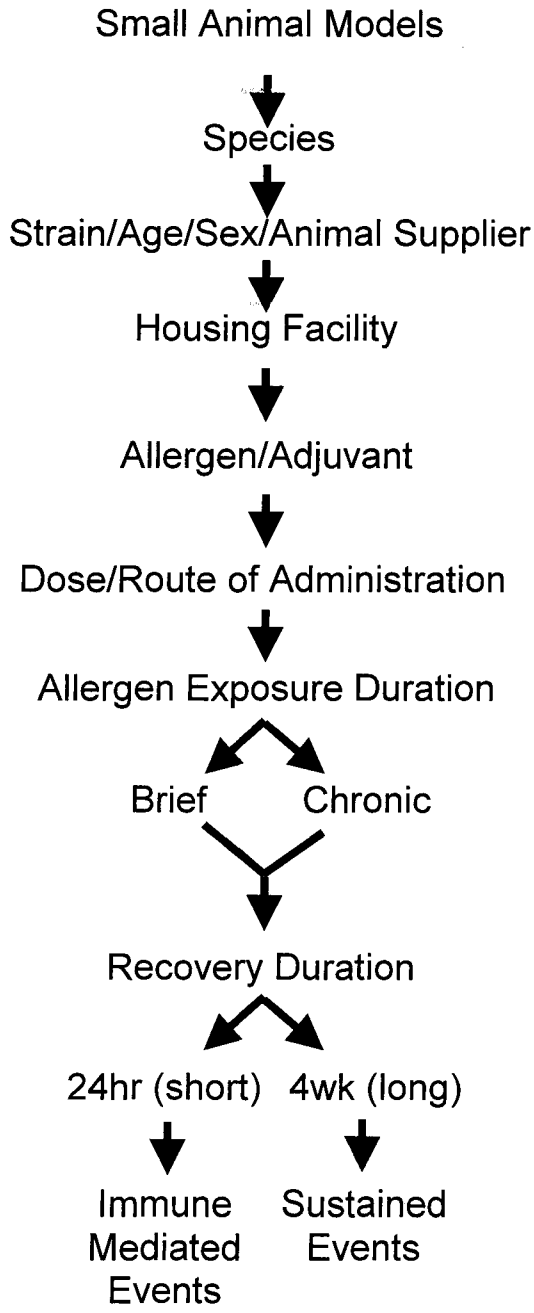

Figure 2: Variables to consider in designing small animal models and interpreting data obtained/published.

Chapter 2

REGIONAL DIFFERENCES IN THE PATTERN OF AIRWAY REMODELING FOLLOWING CHRONIC ALLERGEN EXPOSURE IN MICE

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Jeremy Hirota's contributions:

As primary author I, J. Hirota, was responsible for conceiving, developing, and managing the entire study. R. Ellis was responsible for study coding to ensure blinding for analysis by myself. I collected, fixed, sliced, and stained tissues. Analysis, statistics, figure generation, manuscript preparation, and submission were performed by me with guidance from M.D. Inman. M.D. Inman is the senior author and primary investigator of the lab that the work was completed in.

Regional differences in the pattern of airway remodeling following chronic allergen exposure in mice

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Key Words:
Airway Remodeling, Asthma, Morphometry, Mouse, Chronic Allergen

Abstract

Background : Airway remodeling present in the large airways in asthma or asthma models has been associated with airway dysfunction in humans and mice. It is not clear if airways distal to the large conducting airways have similar degrees of airway remodeling following chronic allergen exposure in mice. Our objective was to test the hypothesis that airway remodeling is heterogeneous by optimizing a morphometric technique for distal airways and applying this to mice following chronic exposure to allergen or saline.

Methods : In this study, BALB/c mice were chronically exposed to intranasal allergen or saline. Lung sections were stained for smooth muscle, collagen, and fibronectin content. Airway morphometric analysis of small (0-50000μm^2), medium (50000μm^2-175000μm^2) and large (>175000μm^2) airways was based on quantifying the area of positive stain in several defined sub-epithelial regions of interest. Optimization of this technique was based on calculating sample sizes required to detect differences between allergen and saline exposed animals.

Results : Following chronic allergen exposure BALB/c mice demonstrate sustained airway hyperresponsiveness. BALB/c mice demonstrate an allergen-induced increase in smooth muscle content throughout all generations of airways, whereas changes in subepithelial collagen and fibronectin content are absent from distal airways.

Conclusions : We demonstrate for the first time, a systematic objective analysis of allergen induced airway remodeling throughout the tracheobronchial tree in mice. Following chronic allergen exposure, at the time of sustained airway dysfunction,

BALB/c mice demonstrate regional differences in the pattern of remodeling. Therefore results obtained from limited regions of lung should not be considered representative of the entire airway tree.

Introduction

The hallmarks of asthma are variable airflow limitation associated with increased airway responsiveness, airway inflammation, and airway remodeling[1-5]. Ongoing airway inflammation and associated airway remodeling are believed to play a role in the development of airway hyperresponsiveness and airflow limitation. The relative contribution of various pathologic components to the increased airway responsiveness is yet to be elucidated, although airway remodeling appears to play a major role[3;5]. In human studies, advances in this area have relied on quantifying established airway remodeling and relating this to airway function measured at the same time[1;3;6]. In animal studies, greater insight is potentially afforded by observing the development of airway remodeling over time and relating this to changes in airway function occurring over the same period[7;8]. We currently use a murine chronic allergen exposure protocol that results in airway remodeling and associated sustained airway dysfunction which persists for up to 8 wks following cessation of allergen[7]. In human and animal approaches, assumptions have been made that measurement of airway remodeling changes at a single, or limited number of airway generations represents the whole lung. While this assumption is necessary when the access to multiple sites is limited (i.e. human biopsy studies), it is unlikely to be valid. In fact, there is evidence that the extent of specific indices of airway remodeling differs depending on the airway generation[9-11].

The involvement of the airways distal to the large conducting airways in respiratory disease, has been debated since Weibel's anatomical classification of small airways as

being less than 2mm in diameter[9;10;12-14]. More recently, the perception of the contribution of the small airways to overall lung resistance has shifted from a silent or quiet zone[15;16], to a more functionally relevant tissue[11;17].

To fully understand the contribution of each airway generation to airway disease we will require methods to assess inflammatory and structural changes throughout these airways. Similar to humans, the distribution of airway remodeling in mice following chronic allergen exposure is currently poorly described. We therefore felt it was prudent to develop and apply objective methods of quantifying airway remodeling throughout the tracheobronchial tree in animal models of allergic airway disease.

It is our hypothesis that quantifying the extent of several indices of airway remodeling in a range of airway calibers will reveal distinct patterns of changes at different levels of the tracheobronchial tree. To test this hypothesis, we present and characterize methods for assessing allergen-induced airway remodeling in the small and medium airways of mice having been subjected to chronic allergen exposure[7]. After optimizing these methods, we report that following chronic allergen exposure, distinct patterns of airway remodeling exist in different sized airways.

Methods

Animals: Female BALB/c wild type mice, aged 10-12 weeks, were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed in environmentally controlled, specific pathogen-free conditions for a one week acclimatization period and throughout the duration of the studies. All procedures were approved by the Animal Research Ethics Board at McMaster University, and conformed to the NIH guidelines for experimental use of animals.

Sensitization and Exposure: Mice were sensitized as described previously by us[7]. Briefly, all mice received intraperitoneal (IP) injections of ovalbumin (OVA) conjugated to aluminium potassium sulfate on Days 1 and 11 and intranasal (IN) OVA on Day 11. Following sensitization, mice were subjected to a chronic allergen exposure protocol (Figure 1). Chronic allergen exposure was comprised of six 2-day periods of intranasal ovalbumin (IN OVA) administration (100 μg in 25 μl saline), each separated by 12 days. Exposures started on Days 19 and 20. Outcome measurements were made four weeks following the final period of allergen exposure and included (i) *in vivo* assessment of airway responsiveness to methacholine, (ii) large airway morphometry as described previously [18] (iii) a novel method for assessing morphometry of small and medium airways.

Airway Responsiveness: Airway responsiveness was measured by total respiratory system resistance (R_{RS}) responses to intravenous saline and increasing doses of

methacholine (MCh) using the FlexiVent ventilator system (n=8 per group). Each mouse was anaesthetized with Avertin (2,2,2-Tribromoethanol, Sigma, Canada) via IP injection at a dose of 240 mg/kg and then underwent tracheostomy with a blunted 18-gauge needle, and then connected to the FlexiVent (SCIREQ, Montreal, Canada) computer-controlled small animal ventilator. Animals were ventilated quasisinusoidally (150 breaths/min, 10 ml/kg, inspiration/expiration ratio of 66.7%, and a pressure limit of 30 cmH_2O). A script for the automated collection of data was then initiated, with the PEEP level set at 2 cmH_20 and default ventilation for mice. After the mouse was stabilized on the ventilator, the internal jugular was cannulated using a 25-gauge needle. Paralysis was achieved using pancuronium (0.03 mg/kg intravenously) to prevent respiratory effort during measurement. To provide a constant volume history, data collection was preceded by a 6 sec inspiration to TLC perturbation (peak amplitude 25 cmH_20). Twenty seconds later the user was prompted to intravenously inject saline then 10, 33, 100, and 330 mg/kg of MCh (ACIC [Can], Brantford, ON, Canada). For each dose, thirteen "QuickSnap-150" perturbations (single inspiration/expiration of 0.4 sec duration with a volume amplitude relative to weight of 10 ml/kg) were performed over a 45 sec period, followed 10 sec later by another 6 sec TLC. After the last dose was complete, the mouse was removed from the ventilator and killed via terminal exsanguinations and subjected to further tissue collection. Airway responsiveness was quantified by the slope of the linear regression between peak respiratory system resistance and the $\log_{10}$ of the MCh dose, using the data from the 10, 33, and 100 μg/kg doses only. Heart rate and oxygen saturation were

monitored via infrared pulse oxymetry (Biox 3700; Ohmeda, Boulder, CO) using a standard ear probe placed over the proximal portion of the mouse's hind limb.

Lung histology: Following *in vivo* assessment of airway responsiveness, lungs were dissected, removed, inflated with 10% formalin with a pressure of 25 cm H_2O, ligated at the trachea, and fixed in 10% formalin for 24 hours. Following fixation, the left lung was isolated and bisected into superior and inferior segments (Figure 2). The inferior portion of the left lobe was embedded with the bisected face down to obtain transverse cross sections of the primary bronchus for large airway morphometry. The superior portion of the left lobe was subjected to a sagittal cut and embedded with the sagittal face down for airway morphometry of airways distal to the primary bronchus (Figure 2). Both superior and inferior lung portions were embedded in the same paraffin wax tissue block, and rough cut to expose a smooth tissue surface. Three micron thick sections were stained with Picrosirius Red (PSR) for assessing the presence of collagen. Further sections were immunostained using monoclonal antibodies against α-smooth muscle actin (α-SMA)(Clone 1A4, Dako, Denmark) and fibronectin (Clone 10, BD Biosciences, Canada)

Lung morphometry: All tissue sections were viewed and images collected under 20X objective magnification light microscopy (Olympus BX40; Carsen Group Inc., Markham Ontario). A customized digital image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, Ontario, Canada) with an attached digital pen and drawing tablet was used to collect and analyze images. Airways that satisfied the

following criteria were included for airway analysis: (i) the airway needed to be completely contained in a single microscope field of view (690 μm x 520 μm); (ii) the ratio of the major and minor airway axes needed to be less than 2 (maximum diameter/minimum diameter) to ensure that the airway was not obliquely cut; (iii) the airway perimeter needed to be completely intact. Images of airways that satisfied these criteria were saved as tagged image file format files. Image collection and analysis was performed by two separate individuals; the first individual would collect, code, and determine the size of airways, the second individual would be blinded and analyze the collected coded images as follows. Using the custom digital image analysis system, quantification of the area of positive stain per region of interest was performed for α-SMA, PSR, and fibronectin stained tissues. Areas of airway wall associated with connective tissue from neighbouring vessels were excluded by drawing boundaries for analysis (Figure 3). While viewing the airway of interest, the basal border of the epithelium (corresponding to the basement membrane) was traced. The image with clearly defined boundaries for morphometric analysis was then saved as a new file to be used for all subsequent steps. Using the image file with established basement membrane trace, a 5 um thick region of interest extending from the trace out into the parenchyma was drawn using the digital pen and tablet (Figure 3). The software then calculated the area of stain within the region of interest based on previously determined stain specific colour plane settings. The amount of positive stain area was then expressed as a percentage of the region of interest area. The process was repeated for each airway image captured from the same animal, which were approximately 4 per animal. The

average percent stain for all airways from the same animal was calculated and used for statistical analysis. The analysis on the same airway was repeated for 10, 15, 20, 25, 30, and 35 μm band depths. Medium and small airways were arbitrarily defined by determining the mean airway area of all airways collected. The airways with areas below the mean were defined as small, while airways with areas above the mean were defined as medium. Large airways were collected and analyzed as defined previously [18].

Statistical Analysis: Summary data used in all comparisons are expressed as mean and standard error of the mean (SEM). To determine optimum band depth for detecting airway remodeling changes, we calculated the sample size that would be required to demonstrate observed allergen-induced changes over a range of band depths. This was chosen as a practically useful way of identifying the band depth with optimal signal to noise characteristics. Sample sizes required for comparing two groups were estimated based on the difference of the means between the allergen and control groups and the mean value of the standard deviations at each given band depth. Sample size requirements were based on a Student's t test analysis and calculated with an assumed power of 80% ($\beta = 0.2$) and an α of 0.05. Differences were assumed to be statistically different when the observed p values were less than 0.05.

Results

Airway Responsiveness

Airway function measurements were made two weeks following chronic allergen exposure (Figure 4A). At this time point, significant increases in both airway reactivity and maximum R_{RS} were observed in BALB/c mice as compared to control animals ($p<0.05$; Figure 4B-C). Break point [7] and EC_{50} analysis of methacholine dose response curves revealed no changes in airway sensitivity (data not shown).

Airway Characteristics

Large (primary bronchus) airways used for airway remodeling analysis ranged from 212 760μm^2 to 418 325μm^2 in area. The mean airway area and diameter were 311 035μm^2 and 630μm, respectively. The airway ratio (maximum to minimum diameter) ranged from 1.02 to 1.95.

Airways distal to the primary bronchus used for airway remodeling analysis ranged from 12 269μm^2 to 172 094μm^2 in area. The mean airway area and diameter were 56 543μm^2 and 270μm, respectively. The airway ratio (maximum to minimum diameter) ranged from 1.01 to 1.98. The airways distal to the first generation bronchus were further divided into small (0-50 000μm^2) and medium (50 000μm^2-175 000) airways, based on mean area, for assessment of regional airway remodeling. The mean small and medium airway areas for saline and allergen exposed animals were not significantly different.

Airway remodeling can be detected in airways distal from the primary bronchus of BALB/c mice

Chronic intranasal allergen exposure resulted in a statistically significant increase in α-SMA content in the small and medium airways of BALB/c mice as compared to saline controls (Figure 5A-B). In small airways, the optimal band depth to detect α-SMA changes was 15μm. This conclusion was based on the band width requiring the smallest sample size to detect the allergen-induced change in α-SMA content (Table 1). In medium airways, an allergen induced increase in α-SMA content was detected for band depths ranging from 15-35μm (Figure 5B). In medium airways, the optimal band depth to detect α-SMA content changes was 20μm(Table 1).

Allergen exposure did not result in statistically significant increases in PSR staining in the small airways (Figure 5C). The medium airways demonstrate statistically significant increases in PSR staining at all band depths assessed following chronic allergen exposure (Figure 5D). The optimal band depth to detect PSR changes was 15μm(Table 1).

Allergen exposure did not result in statistically significant increases in fibronectin staining in the small airways (Figure 5E). Statistically significant increases in medium airway fibronectin content were detected following chronic allergen exposure (Figure 5F). The optimal band depth to detect fibronectin changes was 20μm(Table 1).

Regional differences in the pattern of airway remodeling are observed in BALB/c mice following chronic intranasal allergen

The data presented above illustrates differences in airway remodeling between small and medium airways. To further investigate the heterogeneity of airway remodeling we compared remodeling events between large (primary bronchus), medium, and small airways using optimized band depths (see above and ref [18]).

Following chronic allergen exposure the medium airways demonstrated a 2.23 fold increase in smooth muscle content, compared to a 1.76 and 1.37 fold increase in the small and large airways, respectively (Figure 6).

Similarly, there was a 3.31 fold increase in medium airway collagen content, compared to 1.87 and 1.72 fold increase in the small and large airways, respectively (Figure 7).

A 3.25 fold increase in fibronectin staining was observed in the medium airways, compared to 1.71 and 1.44 fold increase in the small and large airways, respectively (Figure 8).

Discussion

Here we demonstrate that regional differences in the pattern of airway remodeling occur in the tracheobronchial tree of mice following chronic allergen exposure. Our morphometric methods for quantifying airway remodeling in mice is the first systematic airway remodeling analysis of the tracheobronchial tree following chronic allergen exposure. These findings are important in demonstrating that insults such as allergen can produce differential effects at different airway levels, which need to be considered when evaluating these animals. Our data therefore support the hypothesis that airway remodeling is heterogeneous in this model of allergen exposure. This emphasizes the importance of treating the tracheobronchial tree as being heterogeneous and argues against approaches with limited scope (eg biopsies) as being reflective of all airway generations.

It is important to emphasize that our decision to divide airways distal to the primary bronchus into small and medium airways is arbitrary and that no anatomical distinction should be inferred. Our division of airways into small, medium, and large groups is required to address the question of heterogeneous airway remodeling. Our findings should therefore be interpreted with this in mind. Precisely defining the airway size/environment required for specific remodeling events or the mechanism underlying these phenomenon was beyond the scope of this manuscript.

As we have previously established morphometric methods for evaluating allergen induced effects only in the large airways[18], we felt it was necessary to extend these techniques to smaller airways. In addition to demonstrating that significant allergen

induced airway remodeling occurs in smaller airways, we show that intranasal allergen exposure results in distinct patterns of remodeling throughout the entire airway tree. The medium airways demonstrate the greatest fold increase in remodeling indices, as compared to the small and large airways. However, whether or not this is the site of the greatest functional consequences of airway remodeling is not known. Clearly, studies aimed at determining the individual contribution of small, medium, and large airways, as well as the specific remodeling events in these airways, to airway dysfunction are required.

We have observed distinct patterns of airway remodeling in different airway generations. While we have clearly demonstrated no statistically significant collagen remodeling in the small airways, it is likely that allergen induced changes in fibronectin would have been statistically significant with a greater sample size (as indicated in the Table). This suggests that studies should be powered according to each of the specific remodeling indices of interest. Failure to do this may result in Type II statistical errors and inappropriate interpretation of results.

Animal research ethics boards require strict guidelines for justifying the number of animals to be used in a given study. Funding agencies are increasingly interested in ensuring that studies are appropriately powered to detect the primary outcome of interest a priori. Our results demonstrate that distinct structural changes occur at different generations of airways, suggesting that group analysis of all airway sizes may mask a signal present in a particular airway size. To appropriately power studies, investigators

should consider the sample size required for analysis of the specific airway size of interest.

The methods presented herein use a customized digital image analysis system, that consists of a CCD camera connected to a microscope and a computer. In addition to the hardware, software capable of detecting user defined colour plane settings is required. We feel that using our validation steps and producing an optimized morphometric technique could be of importance in other research areas including kidney fibrosis, gastrointestinal tract inflammation, and/or vascular biology.

In conclusion we demonstrate that distinct patterns of airway remodeling occur in the tracheobronchial tree of mice following chronic allergen exposure. These results demonstrate that the pathology observed in one area of the lung may not be representative of other regions. Clearly, future studies aimed at exploring structure-function relationships need to consider the heterogeneity of airway remodeling throughout the lung.

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Tables and Figures

Figure 1. Chronic allergen exposure protocol. Sensitization was performed on Day 1 and Day 11. Six 2-day periods of allergen exposure, each separated by 12 days, started on Days 19 and 20. Outcomes were performed 4 wks post chronic allergen exposure.

Figure 2. Depiction of left lobe following inflation and fixation with formalin. The left lobe was bisected to produce superior and inferior portions. The superior half of the left lobe was subjected to a sagittal cut. The superior and inferior portions were embedded in the same tissue block with extreme inferior and superior sagittal faces down (thick lines) and subjected to serially sectioning (fine lines).

Figure 3. Depiction of a small airway captured for analysis. The airway is associated with vessels, which are excluded from morphometric analysis of airway walls. The sub-epithelial basement membrane of the airway wall free from vessel association is traced. A region of interest of defined band depth (5, 10, 15, 20, 25, 30, and 35μm) is projected into the parenchyma from the sub-epithelial basement membrane trace (black lines). The stain of interest (α-SMA) is quantified by the software as a percentage of the total band area for each band depth.

Figure 4. **A)** Airway physiology responses to increasing doses of MCh measured two weeks following chronic exposure to saline (open) or OVA (closed) on FlexiVent ventilator system. BALB/c saline (triangles), BALB/c OVA (squares).

(B) Airway reactivity and **(C)** maximum respiratory resistance values as measured by MCh dose response slope and maximum resistance, respectively for chronic saline (open) or OVA (closed) BALB/c mice

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from corresponding control animals ($p<0.05$).

Figure 5. Morphometric analysis of small and medium airways following chronic exposure to saline (open) or OVA (closed). Morphometric analysis was performed at 5, 10, 15, 20, 25, 30, and 35μm band depths. The stain of interest is expressed as a percentage of total band area. Open bars – saline exposed animals, Closed bars – ovalbumin exposed animals. **A)** Small airway α-SMA staining. **B)** Medium airway α-SMA staining. **C)** Small airway Picrosirius Red (PSR) staining. **D)** Medium airway PSR staining. **E)** Small airway fibronectin staining. **F)** Medium airway fibronectin staining

Data are expressed as mean (SEM); 8 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

** significantly different from corresponding control animals ($p<0.01$)

*** significantly different from corresponding control animals ($p<0.001$)

Figure 6. Morphometric analysis of smooth muscle content in small, medium, and large airways following chronic exposure to saline (open) or ovalbumin (closed). Morphometric analysis for small and medium airways used 15 and 20 μm band depths, respectively. Proximal airways were analyzed as described previously[18]. **A)** Large

airway α-SMA staining. **B)** Medium airway α-SMA staining. **C)** Small airway α-SMA staining. Representative histology images for large, medium, and small airways are located to the right of the figures. Data are expressed as mean (SEM); 8 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

Figure 7. Morphometric analysis of collagen content in small, medium, and large airways following chronic exposure to saline (open) or ovalbumin (closed). Morphometric analysis for small and medium airways used 10 and 15 μm band depths, respectively. Proximal airways were analyzed as described previously[18]. **A)** Large airway PSR staining. **B)** Medium airway PSR staining. **C)** Small airway PSR staining. Representative histology images for large, medium, and small airways are located to the right of the figures. Data are expressed as mean (SEM); 8 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

Figure 8. Morphometric analysis of fibronectin content in small, medium, and large airways following chronic exposure to saline (open) or ovalbumin (closed). Morphometric analysis for small and medium airways used 10 and 20 μm band depths, respectively. Proximal airways were analyzed as described previously[18]. **A)** Large airway fibronectin staining. **B)** Medium airway fibronectin staining. **C)** Small airway fibronectin staining. Representative histology images for large, medium, and small

airways are located to the right of the figures. Data are expressed as mean (SEM); 8 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

Table 1. Mean differences of percentage stain between saline and allergen exposed animals

	Band Depth (μm)						
Stain	**5**	**10**	**15**	**20**	**25**	**30**	**35**
α-SMA							
Small	9.78 (9)	13.09 (5)	13.73 (4)	12.24 (5)	10.56 (6)	9.67 (6)	8.58 (6)
Medium	4.48 (84)	18.35 (6)	26.61 (4)	28.43 (4)	28.41 (4)	26.92 (4)	25.81 (4)
PSR							
Small	3.49 (44)	3.52 (34)	3.36 (20)	2.93 (18)	2.65 (14)	2.49 (12)	2.20 (11)
Medium	18.81 (3)	19.97 (3)	20.16 (3)	19.04 (3)	16.38 (3)	14.76 (3)	13.09 (3)
Fibro							
Small	6.66 (26)	9.67 (10)	9.59 (8)	9.42 (8)	8.76 (7)	8.16 (7)	7.40 (7)
Medium	16.92 (5)	24.66 (3)	29.55 (3)	30.76 (3)	29.88 (4)	28.79 (4)	27.20 (4)

Numbers in each column are absolute differences between mean values of percentage stain for saline and allergen exposed animals with sample size requirements for determining allergen induced effects in parenthesis.

α-SMA - α- smooth muscle actin stain

PSR – Picrosirius red stain

Fibro – Fibronectin stain

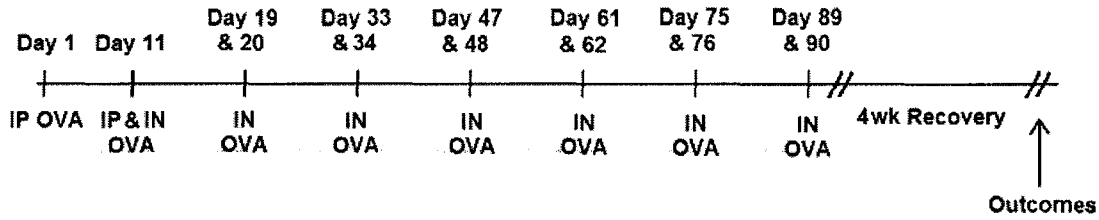
Day 1
Day 11
Day 19 & 20
Day 33 & 34
Day 47 & 48
Day 61 & 62
Day 75 & 76
Day 89 & 90
IP OVA
IP & IN OVA
IN OVA
IN OVA
IN OVA
IN OVA
IN OVA
IN OVA
4wk Recovery
Outcomes

Figure 1:

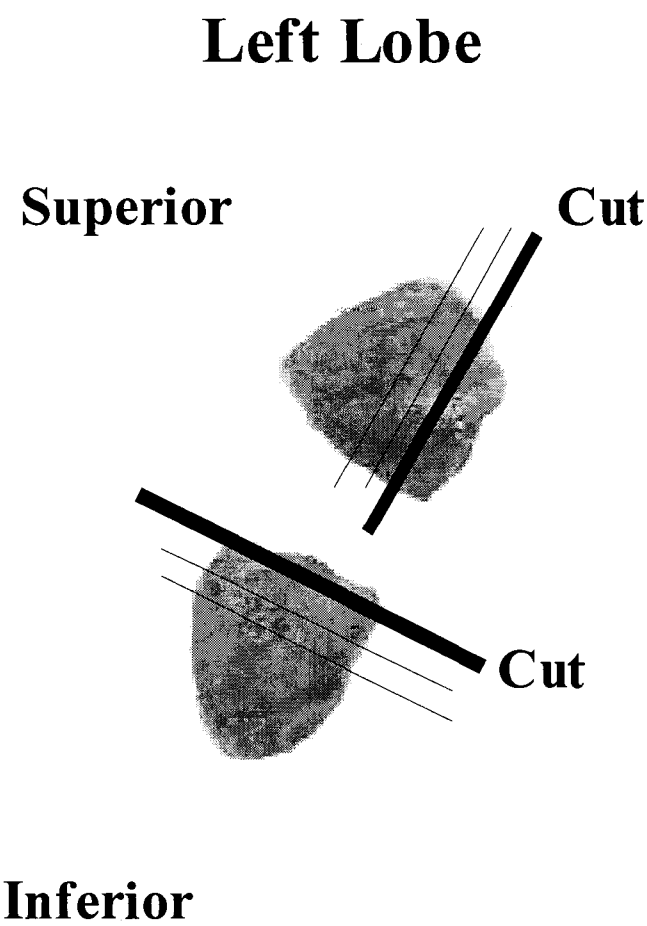

Figure 2

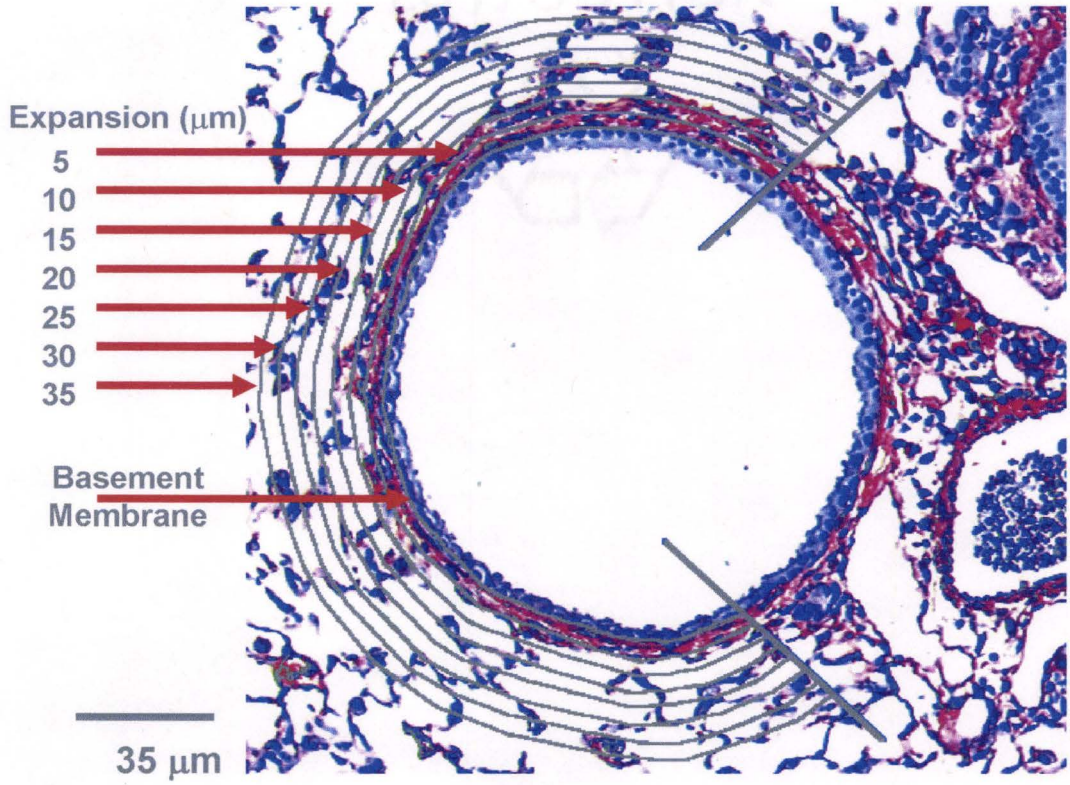

Figure 3

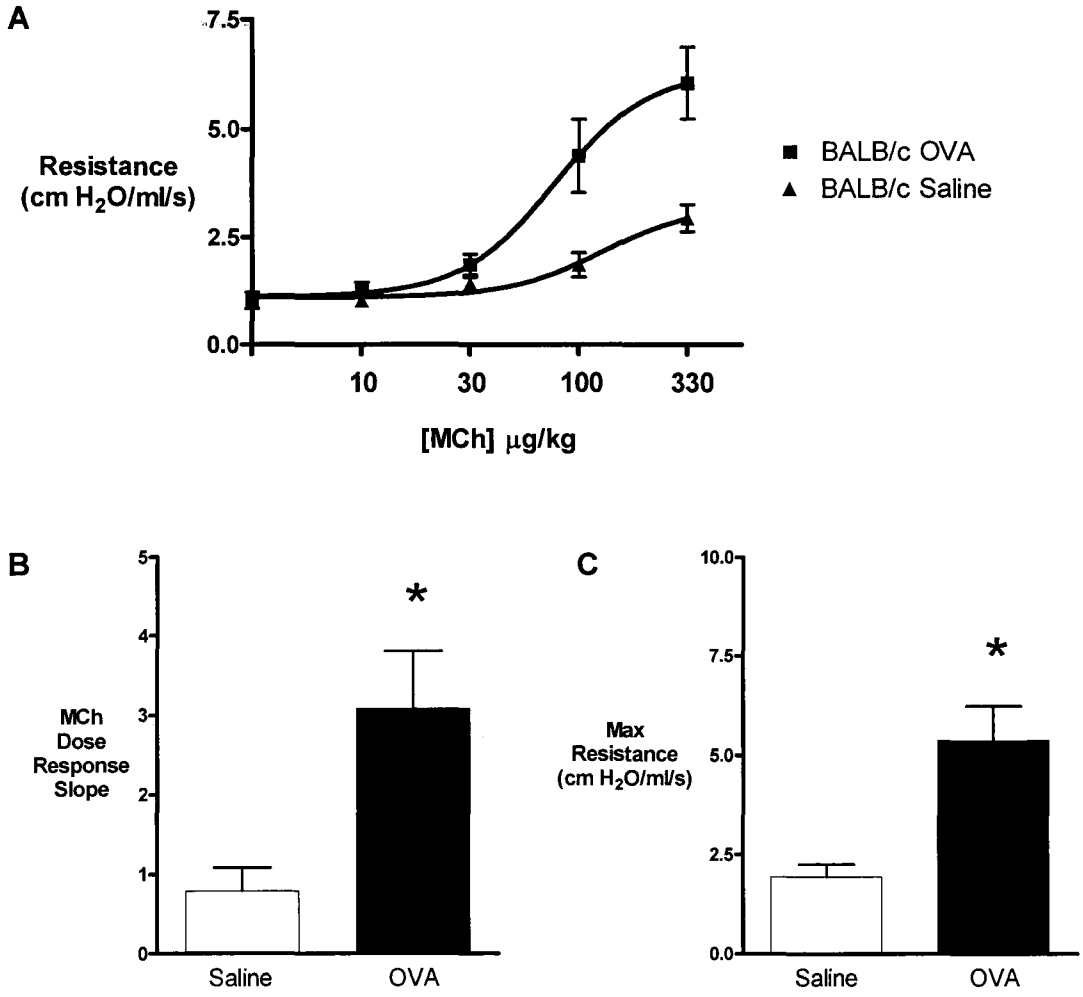
A
Resistance
(cm H2O/ml/s)
7.5
5.0
2.5
0.0
10
30
100
330
[MCh] µg/kg
BALB/c OVA
BALB/c Saline
B
MCh
Dose
Response
Slope
5
4
3
2
1
0
*
Saline
OVA
C
Max
Resistance
(cm H2O/ml/s)
10.0
7.5
5.0
2.5
0.0
*
Saline
OVA

Figure 4

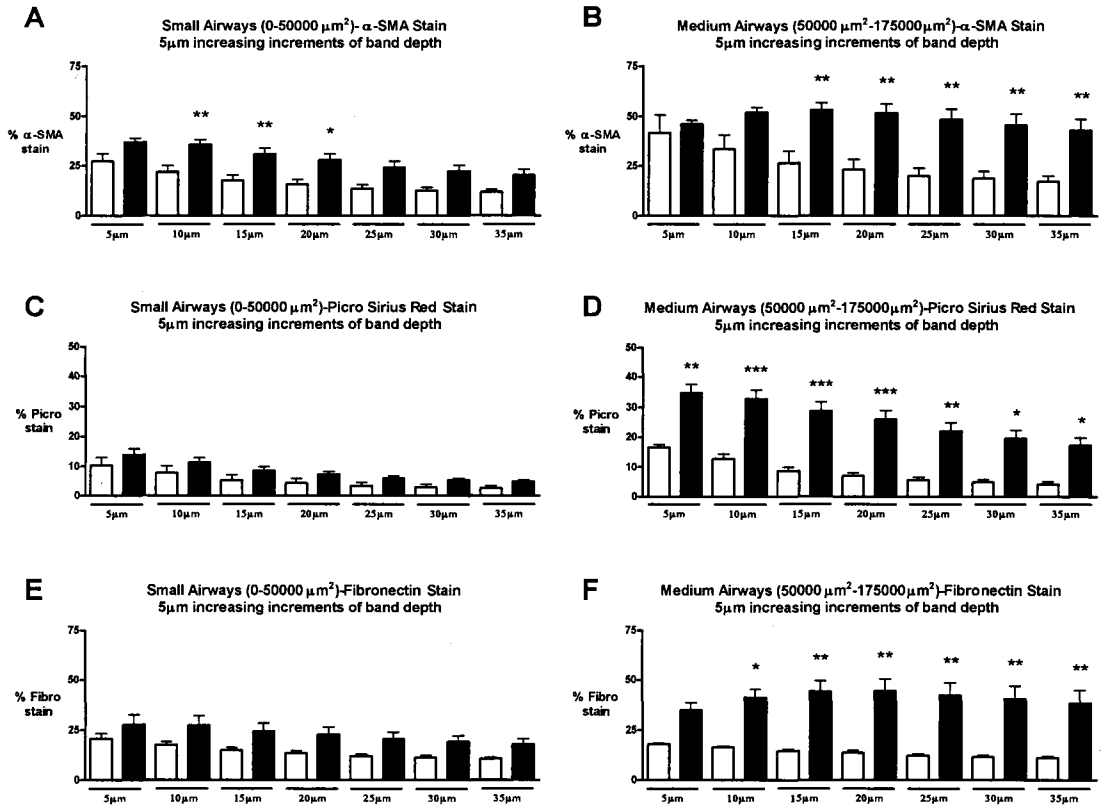
A
Small Airways (0-50000 μm²)- α-SMA Stain
5μm increasing increments of band depth
% α-SMA stain
B
Medium Airways (50000 μm²-175000μm²)-α-SMA Stain
5μm increasing increments of band depth
% α-SMA stain
C
Small Airways (0-50000 μm²)-Picro Sirius Red Stain
5μm increasing increments of band depth
% Picro stain
D
Medium Airways (50000 μm²-175000μm²)-Picro Sirius Red Stain
5μm increasing increments of band depth
% Picro stain
E
Small Airways (0-50000 μm²)-Fibronectin Stain
5μm increasing increments of band depth
% Fibro stain
F
Medium Airways (50000 μm²-175000μm²)-Fibronectin Stain
5μm increasing increments of band depth
% Fibro stain
5μm
10μm
15μm
20μm
25μm
30μm
35μm

Figure 5

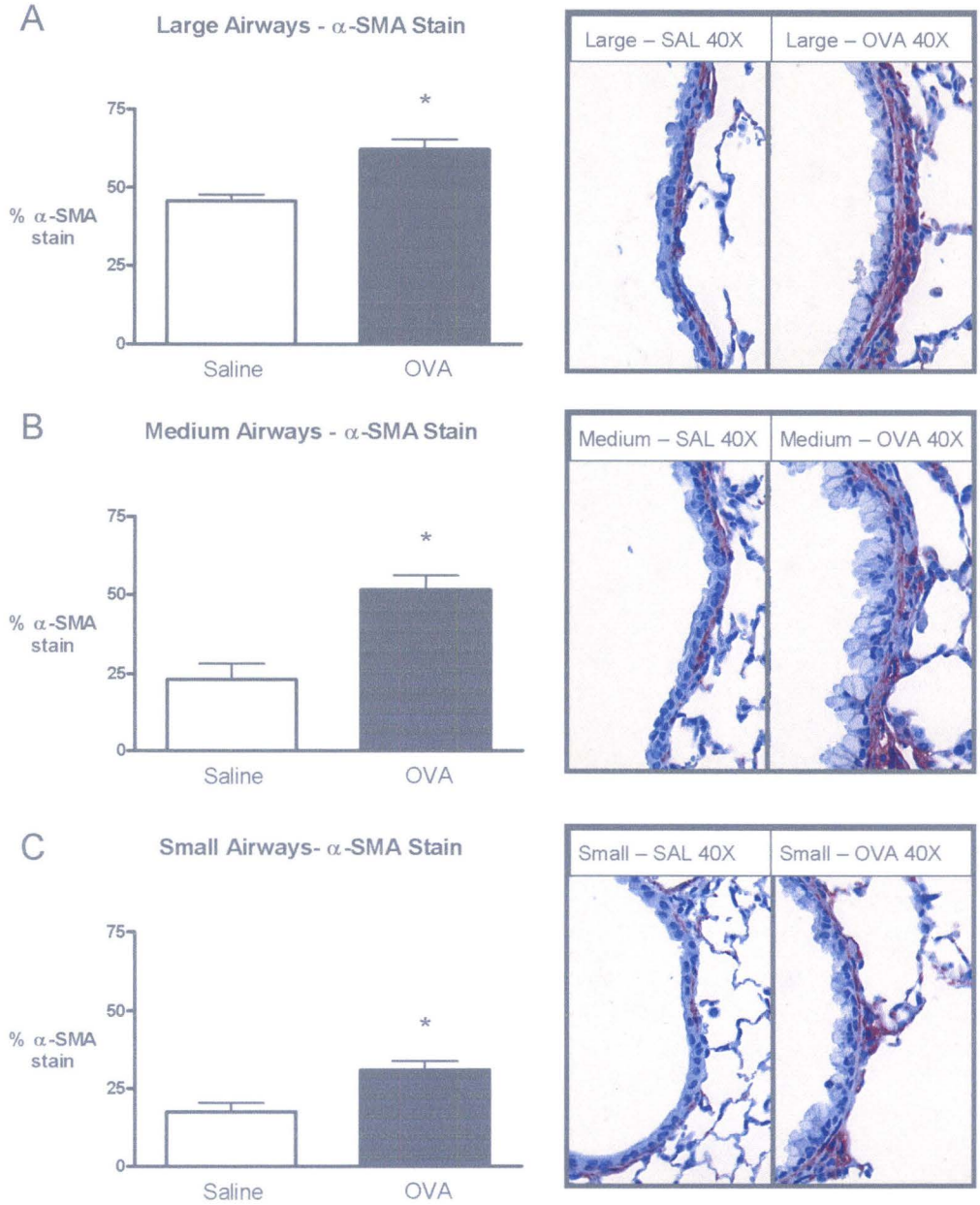
A
Large Airways - α-SMA Stain
% α-SMA stain
75
50
25
0
*
Saline
OVA
Large – SAL 40X
Large – OVA 40X
B
Medium Airways - α-SMA Stain
% α-SMA stain
75
50
25
0
*
Saline
OVA
Medium – SAL 40X
Medium – OVA 40X
C
Small Airways- α-SMA Stain
% α-SMA stain
75
50
25
0
*
Saline
OVA
Small – SAL 40X
Small – OVA 40X

Figure 6

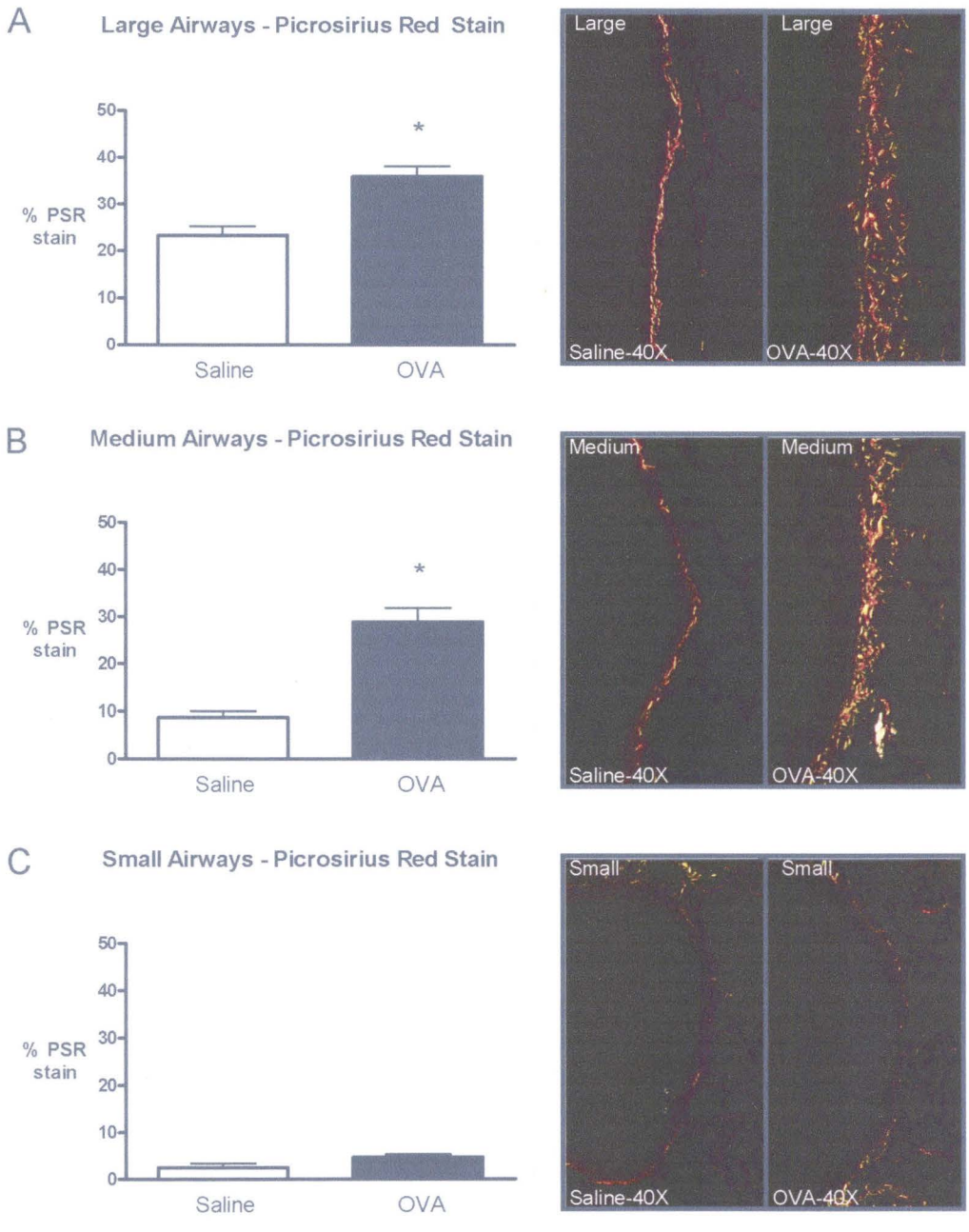
A
Large Airways - Picrosirius Red Stain
% PSR stain
50
40
30
20
10
0
*
Saline
OVA
Large
Large
Saline-40X
OVA-40X
B
Medium Airways - Picrosirius Red Stain
% PSR stain
50
40
30
20
10
0
*
Saline
OVA
Medium
Medium
Saline-40X
OVA-40X
C
Small Airways - Picrosirius Red Stain
% PSR stain
50
40
30
20
10
0
Saline
OVA
Small
Small
Saline-40X
OVA-40X

Figure 7

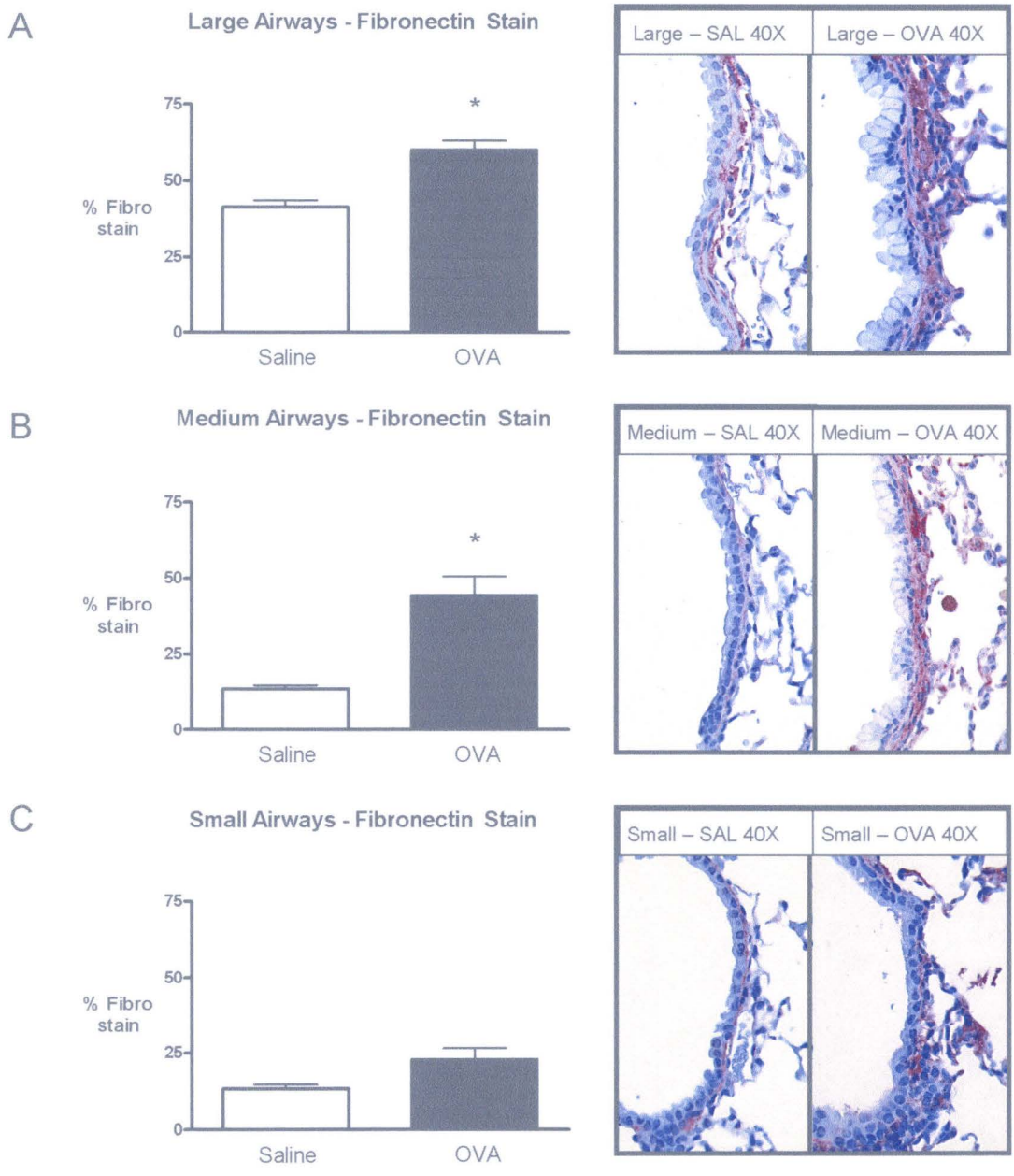
A
Large Airways - Fibronectin Stain
% Fibro stain
75
50
25
0
*
Saline
OVA
Large – SAL 40X
Large – OVA 40X
B
Medium Airways - Fibronectin Stain
% Fibro stain
75
50
25
0
*
Saline
OVA
Medium – SAL 40X
Medium – OVA 40X
C
Small Airways - Fibronectin Stain
% Fibro stain
75
50
25
0
Saline
OVA
Small – SAL 40X
Small – OVA 40X

Figure 8

CHAPTER 3

THE ROLE OF STAT6 AND SMAD2 IN A MODEL OF CHRONIC ALLERGEN EXPOSURE : A MOUSE STRAIN COMPARISON STUDY

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Jeremy Hirota's contributions:

As primary author I, J. Hirota, was responsible for conceiving, developing, and managing the entire study. This included tissue collection, processing, gene expression analysis, immunoblot analysis, immunohistochemistry, and morphometrics. K. Ask was involved in manuscript preparation, procurement of staining and gene expression protocols, and aided in gene expression analysis and figure generation. D. Fritz was involved in optimizing immunoblot protocols. R. Ellis performed tissue slicing for histology. J. Wattie was responsible for animal handling, including allergen exposures and airway physiology measurements. C.D. Richards provided insight into immunoblot methods. R. Labiris was responsible for the animal imaging contained within the manuscript. M. Kolb provided reagents and lab infrastructure for gene expression analysis and manuscript revisions. Analysis, statistics, figure generation, manuscript preparation, and submission were performed by me with guidance from M.D. Inman. M.D. Inman is the senior author and primary investigator of the lab that the work was completed in.

The role of STAT6 and SMAD2 in a model of chronic allergen exposure : A mouse strain comparison study

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Key Words :
Asthma, Airway Remodeling, Th1/Th2 Cells, Transgenic/Knockout Mice

Abstract

Background : Asthma is a disease characterized by variable and reversible airway obstruction and associated with airway inflammation, airway remodeling (including goblet cell hyperplasia, increased collagen deposition, and increased smooth muscle mass), and increased airway responsiveness. It is believed that airway inflammation plays a critical role in the development of airway remodeling, with IL-13 and TGF-β1 pathways strongly associated with disease progression. Mouse models of asthma are capable of recapitulating some components of asthma and have been used to look at both IL-13 and TGF-β1 pathways, which use STAT6 and SMAD2 signaling molecules, respectively.

Objectives : Using brief and chronic models of allergen exposure we utilized BALB/c and C57Bl/6 to explore the hypothesis that observed differences in responses to allergen between these mouse strains will involve fundamental differences in IL-13 and TGF-β1 responses.

Methods : The following outcome measurements were performed: airway physiology, bronchoalveolar lavage cell counts/cytokine analysis, histology, immunoblots, and gene expression assays.

Results : We demonstrate in BALB/c mice, an IL-13 dependent phosphorylation of STAT6, nuclear localized in inflammatory cells, which is associated with indices of airway remodeling and development of airway dysfunction. In BALB/c mice, phosphorylation of SMAD2 is delayed relative to STAT6 activation and also involves an IL-13 dependent mechanism. In contrast, despite an allergen induced increase in IL-4,

IL-13, and eosinophils, C57Bl/6 demonstrate a reduced and distinct pattern of phosphorylated STAT6, no SMAD2 phosphorylation changes, and fail to develop indices of remodeling or changes in airway function.

Conclusion : The activation of signaling pathways and nuclear translocation of signaling molecules downstream of IL-13 and TGF-β1 further support the central role of these molecules in the pathology and dysfunction in animal models of asthma. Activation of signaling pathways downstream from IL-13 and TGF-β1 may be more relevant in disease progression than elevations in airway inflammation alone.

Introduction

Asthma is a disease characterized by variable and reversible airway obstruction and associated with airway inflammation, airway remodeling, and airway hyperresponsiveness (AHR). While the precise mechanisms are poorly understood, it is believed that AHR in asthma is secondary to both inflammatory and remodeling changes in the airways(1). It is further believed that airway inflammation plays a critical role in the development of airway remodeling, although the relative contribution and temporal actions of various mediators or cells (e.g. IL-13, TGF-β1, eosinophils) remains to be determined. Furthermore, with currently available therapies more efficacious in treating the inflammation as compared to established airway remodeling, there remains a need to understand the pathways involved in development and maintenance of the remodeled airway.

IL-13 is a central mediator in allergic asthma and has been implicated in numerous ways with the development of pathology and functional changes(2). The pleiotropic nature of IL-13 with respect to the development of goblet cell hyperplasia(2,3), increased responsiveness of smooth muscle(4), and induction of the profibrotic mediator TGF-β1(5) make it a promising target for therapies. We and others have demonstrated that IL-13 is crucial for the development of several indices of airway remodeling and the associated sustained AHR following chronic allergen exposure in mice(2,3). Based on this pre-clinical evidence, there is much interest in IL-13 signaling as a site of intervention in the treatment of asthma.

TGF-β1 is also increased in asthmatic airways and has been positively correlated with basement membrane thickness in bronchial mucosal biopsies(6). Additionally, it has been demonstrated that in bronchial epithelial cells differential expression of TGF-β1 is observed in asthmatics as compared to normal controls(7). In asthmatic airways it is believed that TGF-β1 may play dual roles as an anti-inflammatory(7) and a wound healing mediator(8). In airway disease models, TGF-β1 has been directly implicated in fibrosis(9) and is involved in the fibrotic remodeling observed in mouse models of chronic allergen exposure(10).

Recent evidence suggests that IL-13 signaling through the IL-13 Rα2 chain up-regulates TGF-β1, implying that IL-13 actions may precede those of TGF-β1(11). Indeed, Elias and colleagues have previously demonstrated that in IL-13 transgenic mice, IL-13 mediated up-regulation of active TGF-β1 and this was associated with airway fibrosis(5).

Given the evidence that supports both IL-13 and TGF-β1 in the development of airway remodeling and associated AHR, we sought to investigate their roles in a murine model of allergen exposure. We and others have observed that remodeling and airway AHR varies markedly between mouse strains(12). Specifically we have observed that BALB/c, but not C57Bl/6 mice, develop airway hyperresponsiveness following brief or chronic allergen exposure. Here, we explore the hypothesis that the difference in responses to allergen between these mouse strains will involve fundamental differences in IL-13 and TGF-β1 responses. To test this hypothesis we looked at brief and chronic

allergen exposure at multiple time-points in two genetically distinct, commonly used inbred mouse strains, BALB/c and C57Bl/6.

Methods

Animals: Female BALB/c and C57Bl/6 wild type mice, aged 8-10 weeks, were purchased from Harlan Sprague Dawley (Indianapolis, IN). Female mice of a BALB/c background and similar age, deficient in IL-4 (IL-4 -/-) or IL-13 (IL-13 -/-) were obtained from The Jackson Laboratory or kindly provided by Dr. Andrew McKenzie at the Medical Research Council Laboratory of Molecular Biology, Cambridge, UK, respectively. All mice were housed in environmentally controlled, specific pathogen-free conditions for a one week acclimatization period and throughout the duration of the studies. All procedures were approved by the Animal Research Ethics Board at McMaster University, and conformed to the NIH guidelines for experimental use of animals.

Sensitization and Exposure: Mice were sensitized as described previously(13). Briefly, all mice received intraperitoneal (IP) injections of ovalbumin (OVA) conjugated to aluminium potassium sulfate on Days 1 and 11 and intranasal (IN) OVA on Day 11. Following sensitization, mice were subjected to an allergen exposure protocol with multiple time points for outcome measurements (Figure 1). Brief allergen exposure was comprised of a single 2-day period of intranasal ovalbumin (IN OVA) administration (100 μg in 25 μl saline) under gaseous anesthesia, with ventilation rate visually monitored between strains to ensure equal plane of anesthesia before IN exposure. Chronic allergen exposure was comprised of six 2-day periods of IN OVA administration each separated by 12 days. Exposures started on Days 19 and 20.

Outcome measurements were made at the following time points; (i) 24hrs after a single 2-day period of allergen exposure (Day 21), (ii) 2wks following a single 2-day period of allergen exposure (Day 34), (iii) 2wks following the complete chronic allergen exposure protocol (Day 104), and (iv) 4wks following the complete chronic allergen exposure (Day 118).

Airway Responsiveness: Airway responsiveness was measured by total respiratory system resistance (R_{RS}) responses to intravenous saline and increasing doses of methacholine (MCh) using the Flow Interrupter ventilator system (n=8 per group) (13). Airway physiology measurements were made on mice 24 hrs after a single period of allergen exposure and 4 wks following the chronic allergen exposure protocol.

BAL and Serum analysis: BAL cell differential analysis was performed as previously described(13). Blood was collected by cardiac puncture. ELISAs were performed for IL-4, IL-13, IFN-γ, and active TGF-β1 in BAL supernatant and IgE levels in serum (BD Biosciences; San Diego, CA and R&D Systems; Minneapolis, MN).

Lung histology: Lungs were dissected and left lobes inflated with 10% formalin with a pressure of 25 cm H_2O, ligated at the trachea, and fixed in 10% formalin for 24 hours. Three micron thick sections were stained with Mayer's Hemotoxylin/Eosin (H&E), Periodic Acid-Schiff (PAS), and Picrosirius Red (PSR), for assessing the presence of tissue eosinophils, goblet cells, and collagen, respectively. Sections were immunostained

using antibodies against α-smooth muscle actin (α-SMA) (Dako, Denmark), phospho-SMAD2 (pSMAD2) (Cell Signaling Technology Inc, Danvers, Mass), and phospho-STAT6 (pSTAT6) (Abcam, Cambridge, Mass). All immunostains required blockage of endogenous peroxidase with a 1.5% H_2O_2/methanol solution for 30 min prior to antigen retrieval in citrate buffer (pH 6.0) in a steamer at 90°C for 20 min followed by cooling of slides to room temperature for 20 min. Blocking was performed with 1% normal swine serum for 30 min at room temperature and all subsequent reagents were dissolved in this solution. α-SMA, pSMAD2, and pSTAT6 primary antibodies were diluted at 1:100, 1:1000, 1:100, respectively and incubated overnight at 4°C. The next morning extensive washes with TRIS buffer (pH 7.6) were performed to remove primary antibodies. In between each addition of reagent, extensive washes with TRIS were performed with squeeze bottles. Biotinylated secondary antibodies were added at 1:100 concentrations, while streptavidin peroxidase was added at 1:600 concentration. Colour development was performed by placing slides into a 0.4% aminoethylcarbazole/dimethylformamide solution for 30 minutes, initiated by the addition of 4 drops of H_2O_2. Counterstaining was performed with Mayer's Hematoxylin.

Lung morphometry: PAS, PSR, α-SMA stained tissue sections were viewed and images collected under 20X objective magnification light microscopy (Olympus BX40; Carsen Group Inc., Markham Ontario). For quantifying the stain, a customized digital image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, Ontario, Canada) with an attached digital pen and drawing tablet was used to collect and

analyze images as described previously(14). H&E stained tissue sections were viewed and images collected under 40X objective. Eosinophils were expressed as number/area in a 50μm band extending from the basement membrane of the large primary bronchus as previously described(13). pSMAD2 and pSTAT6 stained tissue sections were viewed and collected under 40X objective. Quantification of pSMAD2 and pSTAT6 positively stained cells was expressed as the percentage of positively stained cells (positive cells/total cells *100).

Immunoblots: Please see details in online supplement. (in this thesis - located after entire manuscript/references/figure legends/figures)

Gene Expression Analysis: Please see details in online supplement. (in this thesis - located after entire manuscript/references/figure legends/figures)

Lung deposition of intranasal solutions in BALB/c and C57Bl/6 assessed by positron emission tomography/computed tomography (PET/CT): Please see details in online supplement. (in this thesis - located after entire manuscript/references/figure legends/figures)

Statistical Analysis: Summary data used in all comparisons are expressed as mean and standard error of the mean (SEM). Comparisons between saline control and OVA exposed mice for each were made using Student's t tests assuming equal variances.

Differences were assumed to be statistically different when the observed p values were less than 0.05.

Results

BALB/c and C57Bl/6 Inflammatory Profiles Following Brief Allergen Exposure

Consistent with the development of an allergic phenotype, both BALB/c and C57Bl/6 mice demonstrated significant increases in serum IgE levels following sensitization and brief exposure to OVA ($p<0.05$; Online Supplement Figure 1a). Allergen induced elevations of the Th2 cytokines, IL-4 and IL-13, were detected in BAL fluid following brief allergen exposure in both BALB/c and C57Bl/6 mice ($p<0.05$; Online Supplement Figure 1b-c). No detectable changes of the Th1 cytokine, IFN-γ, were observed in the BAL fluid for either strain of mice (Online Supplement Figure 1d). Increases in BAL and tissue eosinophils were also observed in both BALB/c and C57Bl/6 mice ($p<0.05$; Online Supplement Figure 1e-f). Active levels of TGF-β1 were assessed by ELISA and were below detection levels in both strains of mice (data not shown). For each inflammatory mediator/marker assessed, BALB/c mice demonstrated a trend for a greater response as compared to C57Bl/6 mice, although this was not statistically significant for any mediator assessed.

Lung deposition of intranasal solutions in BALB/c and C57Bl/6 assessed by positron emission tomography/computed tomography (PET/CT)

To confirm that our observed strain dependent results were the product of similar distribution patterns of IN OVA, we performed in vivo imaging of IN 18-fluorodeoxyglucose (18-FDG) using PET/CT administered in identical fashion to OVA. We performed in vivo whole body PET/CT imaging immediately after intranasal

instillation of 25μl of 18-FDG (Figure 2). The mean percentage of total radioactivity (adjusted for amount of radioactivity delivered and body weight) in BALB/c mice was 42.25% with SD of 11.19%, while in C57Bl/6 mice the detected radioactivity was 32.29% with SD of 16.64%. No statistically significant difference in distribution of IN 18-FDG was observed ($p>0.05$, $n=5$ for each group). To detect statistical significance a sample size of 31 would be required based on the means and standard deviations obtained from this group of animals.

Activation of STAT6 and SMAD2 Pathways Following Brief Allergen Exposure

We and others have shown that IL-4 and IL-13 are necessary for development of airway hyperresponsiveness and indices of airway remodeling(2,3,15). Here, we determined the activity of the common signaling pathway of IL-4 and IL-13 by investigating the phosphorylation of STAT6 (Figure 3a-g). Morphology and morphometry were used for qualitative and quantitative assessment of pSTAT6, respectively.

We observed allergen induced increases in BAL IL-4 and IL-13 in both BALB/c and C57Bl/6 mice, with a greater response in former strain. Despite elevations in IL-4 and IL-13 observed in both strains, activation of the STAT6 pathway, as indicated by pSTAT6 staining, was observed 24hrs following brief allergen exposure in BALB/c, but not C57Bl/6 mice ($p<0.05$; Figure 3a). The increased BALB/c pSTAT6 staining was nuclear localized in small, nuclear dense, inflammatory cells with lymphocyte morphology ($p<0.05$; Figure 3c).

The activation of the STAT6 pathway in BALB/c mice was sustained as evidenced by significant increases in pSTAT6 stained positive cells 2wks following brief allergen exposure ($p<0.05$; Figure 3a). Consistent with the data obtained at the earlier time point, positive staining cells 2wks after allergen exposure in BALB/c mice demonstrated nuclear localization of pSTAT6 in small, nuclear dense, inflammatory cells with lymphocyte morphology (Figure 3d).

C57Bl/6 mice demonstrated no significant increases in pSTAT6 staining at either the 24h or 2wk time points ($p<0.05$; Figure 3a), although some staining was observed in both control and allergen exposed animals that appeared to be membrane localized in large, single nucleated, inflammatory cells with macrophage morphology (Figure 3e,g).

TGF-β1 mRNA or active protein levels are not absolute markers of TGF-β1 activation(16). Recently it has been suggested that SMAD2 phosphorylation is a more relevant measure of TGF-β1 activation(17,18). We therefore determined the activity of the TGF-β1 pathway by investigating the phosphorylation of SMAD2 (Figure 3h).

Immediately following brief allergen exposure, no detectable increases in pSMAD2 levels were observed in BALB/c or C57Bl/6 mice (Figure 3h). A delayed elevation in nuclear localized pSMAD2 levels was observed 2wks following brief allergen exposure in BALB/c, but not C57Bl/6 mice (Figure 3k). The pSMAD2 staining in BALB/c mice was apparent in epithelial, smooth muscle, and inflammatory cells.

To support and confirm our immunohistochemistry of pSTAT6 staining, we performed immunoblot analysis on tissues from BALB/c and C57Bl/6 mice 24hrs following brief allergen exposure (Figure 4a-c). We demonstrate in whole lung

homogenate, pSTAT6 is elevated in BALB/c mice following allergen exposure, but not in C57Bl/6 mice. No allergen induced differences in total STAT6 expression were observed in either BALB/c or C57Bl/6 mice (data not shown).

It has been demonstrated in transgenic mice that TGF-β1 gene expression and production may be downstream of IL-13 signaling(5). We explored this hypothesis and demonstrate that in BALB/c mice, pSTAT6 signaling is associated with TGF-β1 gene expression ($p<0.05$; Online Supplement Figure 2). In C57Bl/6 mice, reduced and distinct patterns of pSTAT6 staining are associated with no changes in TGF-β1 gene production. Finally, the elevation in pSMAD2 levels in BALB/c mice ($p<0.05$; Figure 3h) was consistent with TGF-β1 mRNA transcript levels that were significantly increased ($p<0.05$; Online Supplement Figure 2).

Airway Physiology Following Brief Allergen Exposure

Following a single 2-day period of allergen exposure, airway physiology measurements were performed in BALB/c and C57Bl/6 mice (Figure 5). In contrast to the observation of similar inflammatory responses in both strains, but consistent with the STAT6 signalling, significant increases in airway reactivity to methacholine and maximum induced airway resistance were observed in BALB/c, but not C57Bl/6 mice ($p<0.05$; Figure 5).

Activity of STAT6 and SMAD2 Pathways Following Chronic Allergen Exposure

STAT6 phosphorylation was maintained throughout chronic allergen exposure in BALB/c mice (Figure 6a). Significant increases in positive stained cells were observed in BALB/c mice with the nuclear staining localized to small, nuclear dense, inflammatory cells with lymphocyte morphology ($p<0.05$; Figure 6c). In contrast to earlier time-points, significant increases in pSTAT6 staining were observed in C57Bl/6 mice following chronic allergen exposure ($p<0.05$; Figure 6a). Interestingly, pSTAT6 staining was restricted to large, single nucleated, inflammatory cells with macrophage morphology and was completely cell membrane localized (Figure 6e).

The delayed increase in pSMAD2 elevation in BALB/c mice was still detectable 2wks post cessation of chronic allergen exposure ($p<0.05$; Figure 6f). No elevations in pSMAD2 levels were detected in C57Bl/6 mice following chronic allergen exposure (Figure 6f).

To confirm and support our immunohistochemistry of pSMAD2 staining, we performed immunoblot analysis on BALB/c and C57Bl/6 mice 2wks following chronic allergen exposure. Consistent with our immunohistochemistry, greater elevations in pSMAD2 levels were detected in BALB/c than C57Bl/6 mice (Figure 7a-c).

Structural Airway Changes Following Chronic Allergen Exposure

Despite similar inflammatory events in BALB/c and C57Bl/6 mice, significant changes in airway structure were restricted to the BALB/c mice, consistent with the early changes in STAT6 and SMAD2 signalling in these mice.

Four weeks following chronic allergen exposure, at a time-point when ongoing inflammation had resolved(13), there were significant increases in airway goblet cell number in BALB/c mice, but not C57Bl/6, compared to control animals ($p<0.05$; Figure 8a).

Consistent with the changes in goblet cells, there were significant increases in subepithelial collagen content in BALB/c mice, but not C57Bl/6, compared to control animals ($p<0.05$; Figure 8b) as assessed by quantitative airway morphometry(14).

Finally, and again consistent with other structural changes, there were significant increased in smooth muscle content in BALB/c mice, but not C57Bl/6, compared to control animals ($p<0.05$; Figure 8c) as assessed by quantitative airway morphometry(14).

Airway Physiology Following Chronic Allergen Exposure

Airway function measurements were made four weeks following chronic allergen exposure (Figure 8d), at a time-point when ongoing inflammation had resolved(13). At this time, consistent with the differences in indices of airway remodeling, significant increases in airway responsiveness was observed in BALB/c, but not C57Bl/6 mice, as compared to control animals ($p<0.05$; Figure 8d).

Role of IL-4 and IL-13 in STAT6 and SMAD2 Phosphorylation

BALB/c mice clearly demonstrate phosphorylation of STAT6 and this appears to be associated with the nucleus. Since STAT6 phosphorylation can be the result of either IL-4 or IL-13 activation, we sought to determine the independent contribution of these

cytokines in BALB/c mice using genetically deficient animals at a time-point when pSTAT6 staining was elevated in wild-type animals.

Following brief allergen exposure, OVA exposed BALB/c IL-4 -/- mice demonstrated an increase in pSTAT6 staining similar to wild type OVA exposed mice ($p<0.05$; Figure 9a-d). Conversely, OVA exposed BALB/c IL-13 -/- mice demonstrated significantly reduced pSTAT6 staining as compared to wild type OVA exposed mice ($p<0.05$; Figure 9a-d). The staining in OVA exposed BALB/c IL-13 -/- mice was similar to saline exposed wild type mice (Figure 9d and Figure 3b).

Activation of the SMAD2 pathway can be induced by IL-13(5,11). As such, we set out to determine the contribution of IL-4 and IL-13 in phosphorylation of SMAD2.

Following chronic allergen exposure, OVA exposed BALB/c IL-4 -/- mice maintained detectable levels of pSMAD2 similar to wild-type OVA mice (Figure 9e-h). Conversely, OVA exposed BALB/c IL-13 -/-, demonstrated significantly reduced pSMAD2 staining as compared to wild type OVA animals ($p<0.05$; Figure 9e-h). The staining in OVA exposed BALB/c IL-13 -/- mice was similar to saline exposed wild type mice (Figure 9h and Figure 6g).

Discussion

The mechanisms responsible for airway remodeling and the relative contribution of these structural changes to AHR are poorly understood. Using two genetically distinct strains of mice we demonstrate that activation of signaling mechanisms downstream from inflammatory mediators are more closely associated with the development of airway remodeling and airway dysfunction, as compared to levels of cells and mediators themselves. Specifically, ongoing Th2 inflammation was observed in both BALB/c and C57Bl/6 mice, although only BALB/c mice progressed to develop structural changes and sustained functional changes. We demonstrate that following brief allergen exposure, STAT6 phosphorylation precedes that of SMAD2, that both of these signals are sustained throughout extended periods of chronic allergen exposure, are associated with airway remodeling, and are IL-13 dependent. Finally, we demonstrate that reduced and distinct patterns of STAT6 phosphorylation are observed in C57Bl/6 mice. By observing similar inflammatory profiles in BALB/c and C57Bl/6 mice, despite disparate development of functional and pathological changes, we suggest that the presence of airway inflammation alone may not be sufficient for chronic disease progression in wild-type mice. Rather, our data suggests that activation of signaling pathways downstream from elevations in inflammatory mediators may be more relevant in disease progression and should be targeted for development of novel therapeutics (e.g. STAT6/SMAD2 nuclear localization inhibitors).

Attempts to delineate mechanisms involved in development of airway remodeling commonly employ genetically modified animals. Results of studies with gene deficient

animals should be carefully interpreted as compensatory/developmental problems can never be entirely ruled out(19). In this context, several experiments have investigated the role of STAT6 in murine models of allergen exposure using STAT6 -/- animals(20,21). Seminal work by Kuperman clearly demonstrated that in a brief allergen model with a single exposure period, BALB/c STAT6 -/- mice were protected from development of allergen induced increased airway responsiveness(21). Further, STAT6 -/- mice were resistant to goblet cell metaplasia and mucus production in this single exposure model, although airway eosinophilia was still detected. Interestingly, long term allergen exposure protocols in STAT6 -/- mice may lead to discrepant results; it has been shown that following chronic allergen exposure BALB/c STAT6 -/- mice are indeed capable of developing eosinophilia, epithelial changes, thickening of subepithelium, and increases in AHR(20). These seemingly disparate findings must be considered in determining the role of STAT6 in an experimental model, and lead us to incorporate various time-points in brief and chronic allergen exposure protocols. Using this approach, we demonstrate that early activation of the STAT6 pathway in BALB/c mice is not only sustained following allergen exposure, but also associated with the development of acute inflammatory and chronic remodeling events. Our findings are consistent with the hypotheses that IL-13 is required for the development of transient AHR following brief exposure to allergen(2), and several indices of remodeling, including goblet cell hyperplasia, increase in smooth muscle, and sub-epithelial fibrosis, following chronic allergen exposure(3). Additionally, we present data that strongly suggests that a marked increase in STAT6 phosphorylation during allergen exposure in BALB/c mice is

dependent on IL-13, but not IL-4. Interestingly, when IL-4 is knocked out we see greater STAT6 phosphorylation, which although completely conjecture, may be the result of different binding thermodynamics between IL-4 and IL-13 and their respective receptors and their coupling efficiency to STAT6 (22).

Our strain dependent results could be in part explained by previously described polymorphisms in the IL-4 Rα receptor(23). Webb and colleagues demonstrated that the IL-4 Rα chains in BALB/c and C57Bl/6 mice are different by 9 amino acids. Interestingly, mice carrying the C57Bl/6 sequence of the IL-4 Rα chain developed AHR, eosinophilia, and elevations in IgE levels that were IL-4 dependent, while mice containing the BALB/c sequence of the IL-4 Rα chain were resistant to these changes. Although these data may be somewhat contradictory to our findings, they suggest that additional sequence differences might exist in the signaling pathways of these two strains (e.g. STAT6 or nuclear import factors) that may lead to variations in post-translational modifications(23).

Of particular interest is the initial distribution of IN instilled solutions in the lungs of BALB/c and C57Bl/6 mouse strains. It is possible that time dependent distribution patterns in the lower and upper airways may differ between strains leading to differential development of immune responses associated with lymphatic tissues in these areas. A time course study imaging the distribution of longer lasting radiolabelled compounds (18-FDG half life = 2h) in BALB/c and C57Bl/6 mice may provide insight into early immune responses.

The TGF-β1 pathway is known to be implicated in a variety of fibrotic diseases, and its activity is often demonstrated by transcript expression or total/active protein levels. Recently it has been suggested that a more suitable marker for TGF-β1 activity is the phosphorylation of its downstream effector, SMAD2(17,18). Consistent with this, we detected TGF-β1 mRNA in the tissue, but not active protein in the lavage fluid from airways of BALB/c mice following brief allergen exposure (data not shown). Our results suggest that TGF-β1 activity is not immediately detectable following allergen exposure but instead ramps up during recovery or continued allergen exposure. It is tempting to suggest that our data supports the inflammation-damage-repair-remodeling paradigm considered to be responsible for airway remodeling(24) although our data remains correlative at this point. Our data support a link between the STAT6 and SMAD2 pathways, as SMAD2 phosphorylation was dependent on IL-13 in BALB/c mice following allergen exposure, in addition to positive associations between TGF-β1 gene expression and pSTAT6 levels. Although the temporality of our data is consistent with the inflammation/damage/repair/remodeling hypothesis, it is clear that further experiments with specific interventions (e.g. STAT6 inhibitors) are required to test this hypothesis and provide complimentary mechanistic data.

The observations of allergic sensitization, Th2 skewed inflammation, and airway eosinophilia in C57Bl/6 mice that are not followed by structural and functional changes are intriguing. Interestingly, we did not see activation of the STAT6 pathway following brief allergen exposure. Even when the STAT6 pathway was activated following chronic allergen exposure, the signal appeared to be membrane localized and detectable only in a

morphologically distinct cell to that observed in BALB/c mice. It is possible that despite C57Bl/6 mice developing Th2 inflammatory markers, associated downstream events, including goblet cell metaplasia, increased levels of TGFβ1, pSMAD2 and fibrosis require nuclear localization of pSTAT6 in a specific cell type. The distinct patterns of pSTAT6 staining in BALB/c and C57Bl/6 mice suggest intracellular signaling, secondary to increased levels of IL-4/IL-13, differ between these strains. To date, no reports of functional consequences of polymorphisms in STAT6 have been reported for different mouse strains or humans. Additionally the presence of nuclear localization signals within STAT6 and/or the mechanisms responsible for nuclear translocation remain to be determined(25). Clearly, our findings suggest that a better understanding of the mechanisms responsible for interfering with this signaling pathway may uncover novel therapeutic targets.

The contribution of airway inflammation to AHR can be explored in short term allergen exposure models, while to accurately examine the role of airway remodeling we must use chronic exposure models – in the absence of ongoing inflammation. Using both brief and chronic allergen exposure models we demonstrate that prolific Th2 skewed inflammation is associated with AHR in BALB/c mice. Th2 inflammation is accompanied by early activation of the STAT6 pathway, delayed activity of the SMAD2 pathway, and development of indices of airway remodeling and sustained AHR in BALB/c mice. Interestingly, in our model C57Bl/6 mice are resistant to development of airway remodeling and sustained AHR despite prolific Th2 inflammation, perhaps the consequence of an abnormal STAT6 signaling pathway. Mechanisms responsible for

pSTAT6 nuclear translocation may provide a suitable therapeutic target for the treatment of airway inflammation and remodeling.

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Figures

Figure 1: Allergen sensitization and exposure protocol. Mice were sensitized with intraperitoneal ovalbumin (IP OVA) injections on Days 1 and 11 and intranasal (IN) OVA on Day 11. Brief allergen exposure was comprised of a single 2-day period of intranasal ovalbumin (IN OVA) administration (100 μg in 25 μl saline). Chronic allergen exposure was comprised of six 2-day periods of IN OVA administration each separated by 12 days. Exposures started on Days 19 and 20. Control animals received saline instead of OVA.

Figure 2: Representative images from small animal whole body PET/CT imaging of BALB/c and C57Bl/6 mice following intranasal administration of 25ul of 18-fluorodeoxyglucose. Red circles outline distribution of 18-FDG to the lung. A: BALB/c mice, B: C57Bl/6 mice.

Figure 3: STAT6 and SMAD2 phosphorylation following brief allergen exposure. **A** - Morphometric analysis of pSTAT6 staining in mice following brief allergen exposure (positive stained cells / total cell number * 100). **B-G** – Representative samples of pSTAT6 staining following brief allergen exposure. **B** – BALB/c brief saline exposed. **C** – BALB/c brief OVA exposed – 24hrs recovery period. **D** – BALB/c brief OVA exposed – 2wk recovery period. **E** – C57Bl/6 brief saline exposed. **F** – C57Bl/6 brief OVA exposed – 24hrs recovery. **G** – C57Bl/6 brief OVA exposed – 2wk recovery. **H** - Morphometric analysis of pSMAD2 staining in mice following brief allergen exposure

(positive stained cells / total cell number * 100). **I-N** – Representative samples of pSMAD2 staining following brief allergen exposure. **I** – BALB/c brief saline exposed. **J** – BALB/c brief OVA exposed – 24hrs recovery period. **K** – BALB/c brief OVA exposed – 2wk recovery period. **L** – C57Bl/6 brief saline exposed. **M** – C57Bl/6 brief OVA exposed – 24hrs recovery. **N** – C57Bl/6 brief OVA exposed – 2wk recovery.

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

Figure 4: Immunoblot for pSTAT6 in brief allergen exposed (24hrs recovery) BALB/c and C57Bl/6 mice. A - pSTAT6. B - Actin. C - Mean normalization (n=2) of pSTAT6 signal to actin level using the densitometry application in BioRad Quantity One Software.

Lane 1: BALB/c Brief SAL

Lane 2: BALB/c Brief SAL

Lane 3: BALB/c Brief OVA

Lane 4: BALB/c Brief OVA

Lane 5: C57Bl/6 Brief SAL

Lane 6: C57Bl/6 Brief SAL

Lane 7: C57Bl/6 Brief OVA

Lane 8: C57Bl/6 Brief OVA

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Figure 5: Airway physiology in mice following brief allergen exposure. Dose response curve to saline and increasing doses of intravenous methacholine.

BALB/c Saline – solid black circle

BALB/c OVA – open white circle

C57Bl/6 Saline – solid black square

C57Bl/6 OVA – open black square

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

Figure 6: STAT6 and SMAD2 phosphorylation following chronic allergen exposure. **A** - Morphometric analysis of pSTAT6 staining in mice following brief allergen exposure (positive stained cells / total cell number * 100). **B-E** – Representative samples of pSTAT6 staining following chronic allergen exposure. **B** – BALB/c chronic saline exposed. **C** – BALB/c chronic OVA exposed – 2wk recovery period. **D** – C57Bl/6 chronic saline exposed. **E** – C57Bl/6 chronic OVA exposed – 2wk recovery **F** - Morphometric analysis of pSMAD2 staining in mice following chronic allergen exposure

(positive stained cells / total cell number * 100). **G-J** – Representative samples of pSMAD2 staining following chronic allergen exposure. **G** – BALB/c chronic saline exposed. **H** – BALB/c chronic OVA exposed – 2wks recovery period. **I** – C57Bl/6 chronic saline exposed. **J** – C57Bl/6 chronic OVA exposed – 2wk recovery.

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

Figure 7: Immunoblot for pSMAD2 in chronic allergen exposed (2wk recovery) BALB/c and C57Bl/6 mice. A - pSMAD2. B - Actin. C - Normalization of pSMAD2 signal to actin level using the densitometry application in BioRad Quantity One Software.

Lane 1: BALB/c Chronic SAL

Lane 2: BALB/c Chronic SAL

Lane 3: BALB/c Chronic OVA

Lane 4: BALB/c Chronic OVA

Lane 5: C57Bl/6 Chronic OVA

Lane 6: C57Bl/6 Chronic OVA

Lane 7: C57Bl/6 Chronic SAL

Lane 8: C57Bl/6 Chronic SAL

Lane 9: TGF-b1 treated rat peritoneal dialysis fluid (+ve control)

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Figure 8: Morphometric analysis of airways and airway physiology responses following chronic allergen exposure. **A** – PAS staining for goblet cells (number of cells/mm basement membrane). **B** – PSR staining for collagen deposition (% stain in region of interest). **C** – α-SMA staining for smooth muscle content (% stain in region of interest).

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from corresponding control animals ($p<0.05$)

D - Airway physiology in mice following chronic allergen exposure. Dose response curve to saline and increasing doses of intravenous methacholine.

BALB/c Saline – solid black circle

BALB/c OVA – open white circle

C57Bl/6 Saline – solid black square

C57Bl/6 OVA – open black square

Figure 9: STAT6 and SMAD2 phosphorylation in IL-4 and IL-13 deficient mice. **A** - Morphometric analysis of pSTAT6 staining in mice following brief allergen exposure (positive stained cells / total cell number * 100). **B-D** – Representative examples of pSTAT6 staining in mice following brief allergen exposure (n=5). Arrows indicate positively stained cells. **B** – BALB/c wild-type mouse. **C** – BALB/c IL-4 -/- mouse. **D** - BALB/c IL-13 -/- mouse. **E** - Morphometric analysis of pSMAD2 staining in mice following chronic allergen exposure (positive stained cells / total cell number * 100). **F-H** – Representative samples of pSMAD2 staining following chronic allergen exposure (n=5). Arrows indicate positively stained cells. **F** – BALB/c wild-type mouse. **G** – BALB/c IL-4 -/- mouse. **H** - BALB/c IL-13 -/- mouse.

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from WT animals ($p<0.05$)

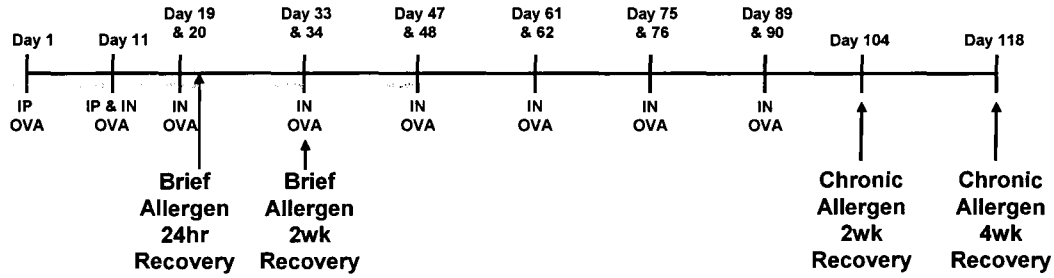

Day 1
Day 11
Day 19 & 20
Day 33 & 34
Day 47 & 48
Day 61 & 62
Day 75 & 76
Day 89 & 90
Day 104
Day 118
IP OVA
IP & IN OVA
IN OVA
IN OVA
IN OVA
IN OVA
IN OVA
IN OVA
Brief Allergen 24hr Recovery
Brief Allergen 2wk Recovery
Chronic Allergen 2wk Recovery
Chronic Allergen 4wk Recovery

Figure 1

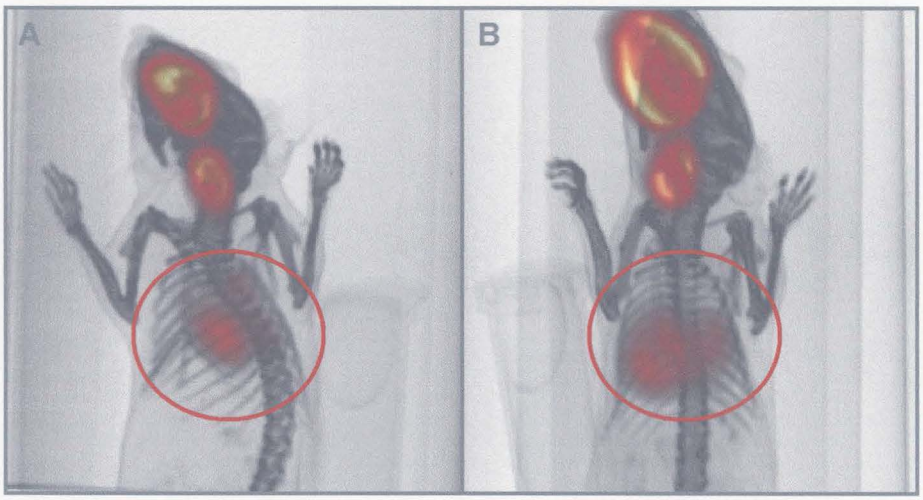


Figure 2

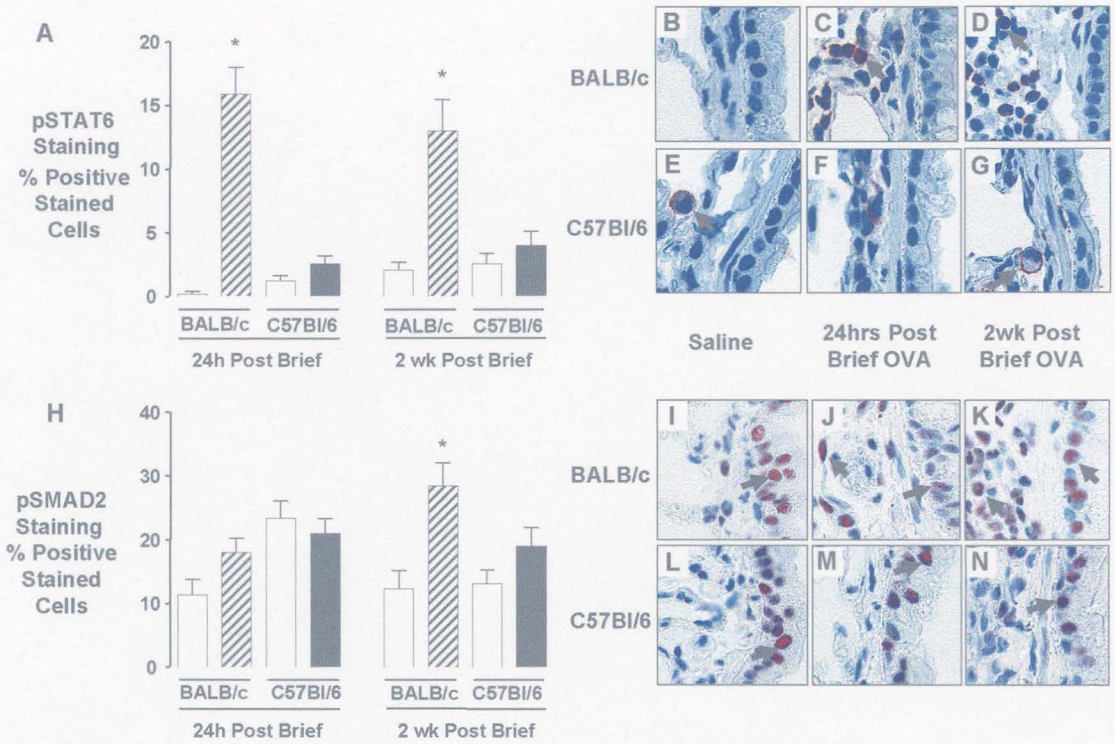

A
pSTAT6 Staining % Positive Stained Cells
20
15
10
5
0
*
*
BALB/c
C57Bl/6
BALB/c
C57Bl/6
24h Post Brief
2 wk Post Brief
B
C
D
E
F
G
BALB/c
C57Bl/6
Saline
24hrs Post Brief OVA
2wk Post Brief OVA
H
pSMAD2 Staining % Positive Stained Cells
40
30
20
10
0
*
BALB/c
C57Bl/6
BALB/c
C57Bl/6
24h Post Brief
2 wk Post Brief
I
J
K
L
M
N
BALB/c
C57Bl/6

Figure 3

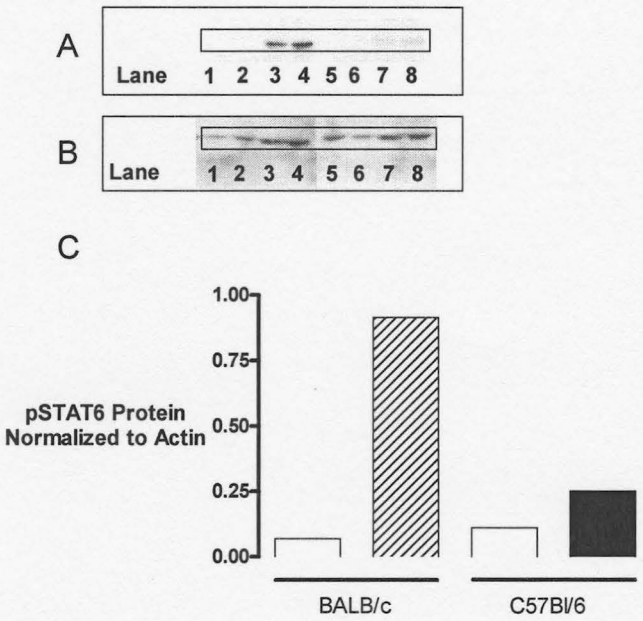

A
Lane 1 2 3 4 5 6 7 8
B
Lane 1 2 3 4 5 6 7 8
C
pSTAT6 Protein Normalized to Actin
1.00
0.75
0.50
0.25
0.00
BALB/c
C57Bl/6

Figure 4

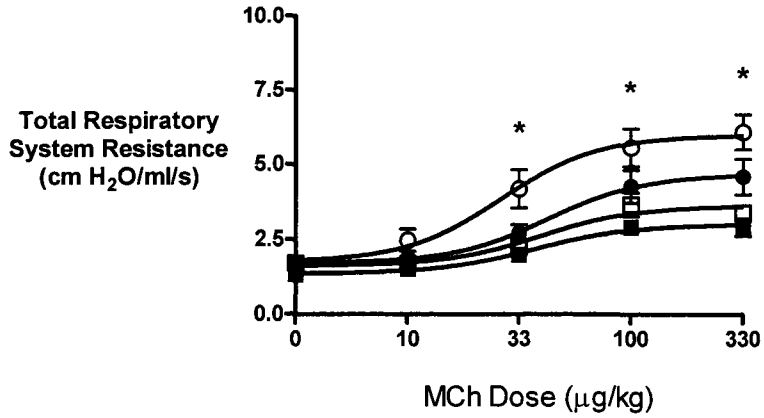

Total Respiratory System Resistance (cm H2O/ml/s)
10.0
7.5
5.0
2.5
0.0
0
10
33
100
330
*
*
*
MCh Dose (μg/kg)

Figure 5

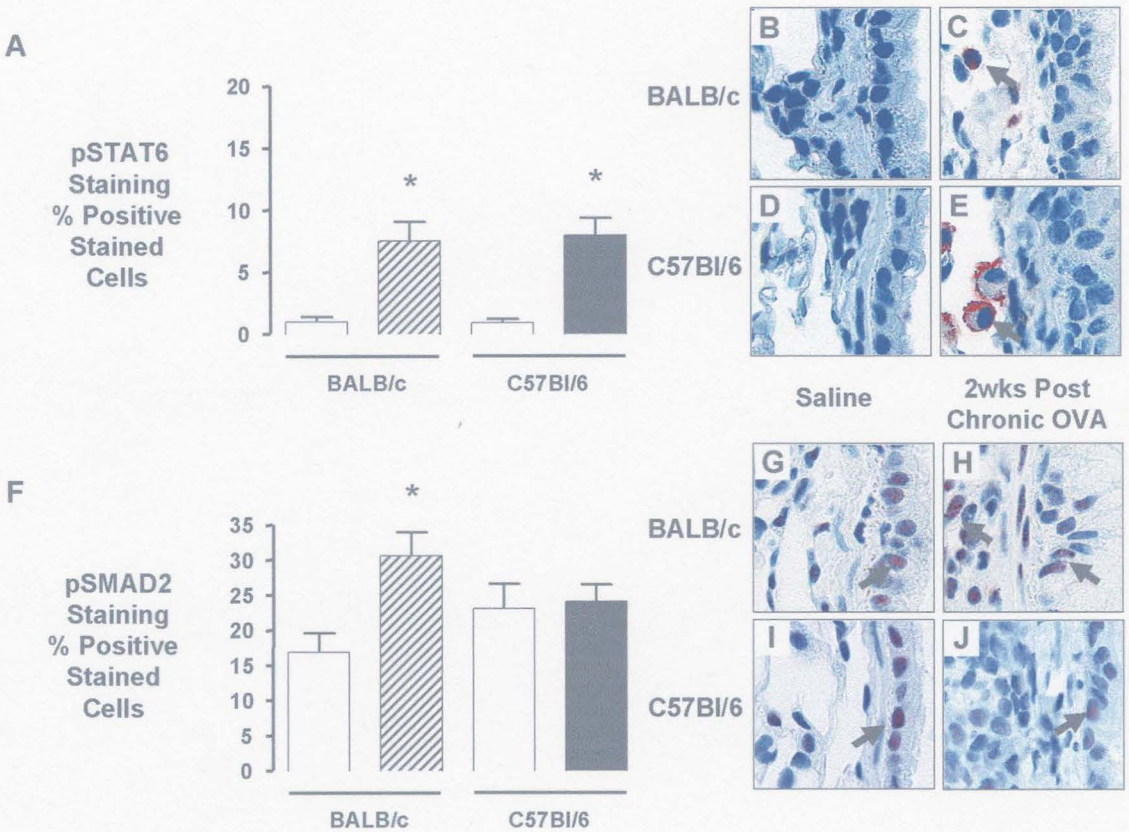

A
pSTAT6 Staining % Positive Stained Cells
20
15
10
5
0
*
*
BALB/c
C57Bl/6
B
C
D
E
BALB/c
C57Bl/6
Saline
2wks Post Chronic OVA
F
pSMAD2 Staining % Positive Stained Cells
35
30
25
20
15
10
5
0
*
BALB/c
C57Bl/6
G
H
I
J
BALB/c
C57Bl/6

Figure 6

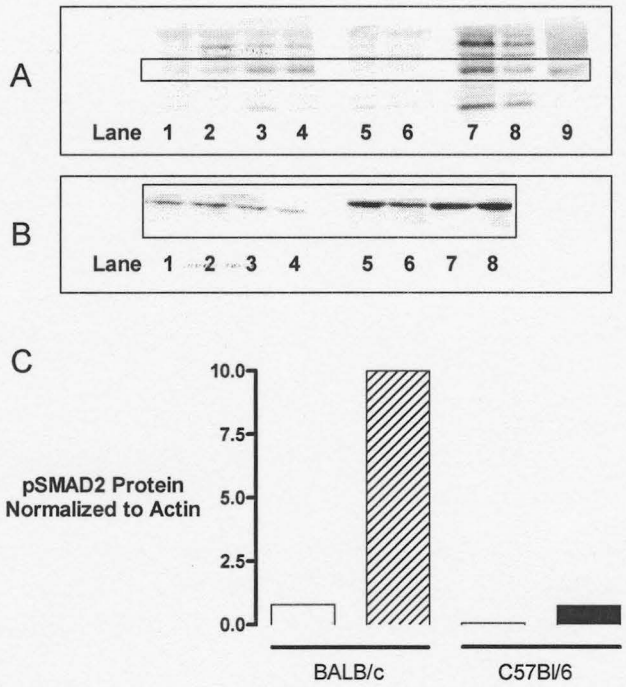

A
Lane 1 2 3 4 5 6 7 8 9
B
Lane 1 2 3 4 5 6 7 8
C
pSMAD2 Protein
Normalized to Actin
10.0
7.5
5.0
2.5
0.0
BALB/c
C57Bl/6

Figure 7

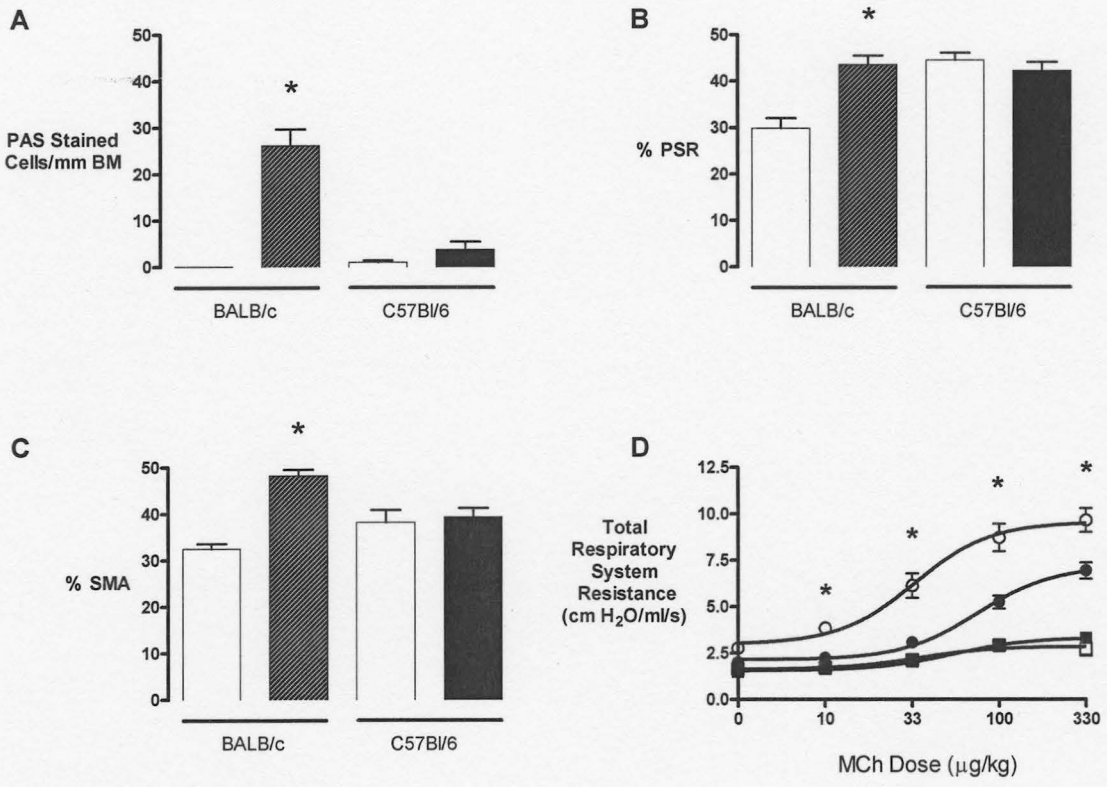

A
PAS Stained Cells/mm BM
50
40
30
20
10
0
*
BALB/c
C57Bl/6
B
% PSR
50
40
30
20
10
0
*
BALB/c
C57Bl/6
C
% SMA
50
40
30
20
10
0
*
BALB/c
C57Bl/6
D
Total Respiratory System Resistance (cm H2O/ml/s)
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
*
0
10
33
100
330
MCh Dose (μg/kg)

Figure 8

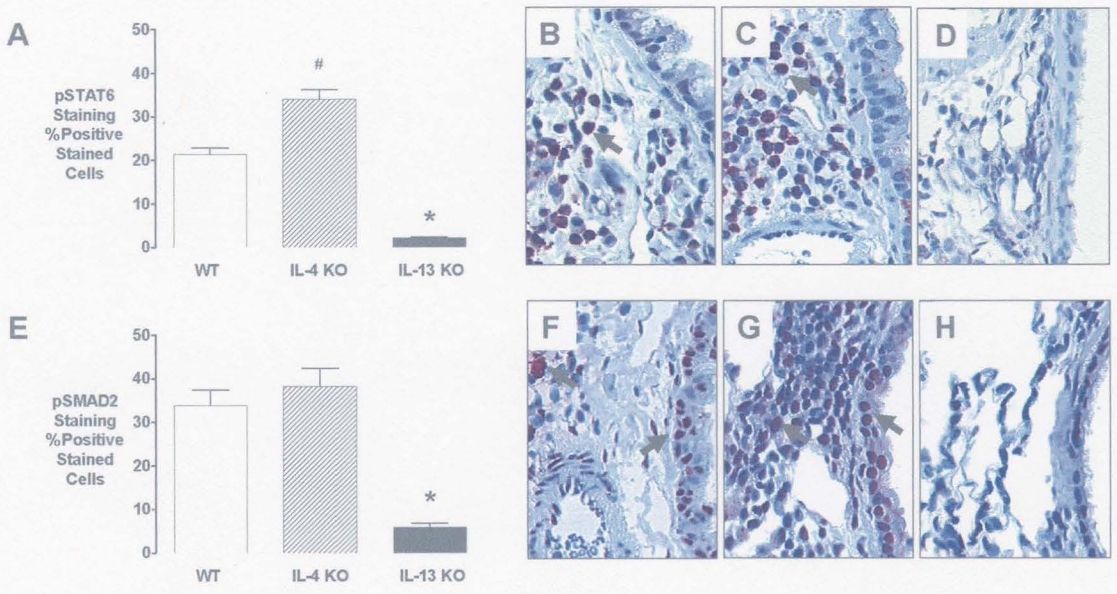

A
pSTAT6 Staining %Positive Stained Cells
50
40
30
20
10
0
#
*
WT
IL-4 KO
IL-13 KO
B
C
D
E
pSMAD2 Staining %Positive Stained Cells
50
40
30
20
10
0
*
WT
IL-4 KO
IL-13 KO
F
G
H

Figure 9

Online Supplement - The role of STAT6 and SMAD2 in a model of chronic allergen exposure : A mouse strain comparison study

Jeremy A. Hirota, Kjetil Ask, Dominik Fritz, Russ Ellis, Jennifer Wattie, Carl D. Richards, Renee Labiris, Martin Kolb, Mark D. Inman

Methods

Immunoblots: Immunoblot primary antibodies (Abs) specific for phosphotyrosine pY641 STAT6 (pSTAT6) and phosphoserine pS465/467 SMAD2 (pSMAD2) were purchased from Cell Signaling Technology Incorporated (Danvers, Mass). Primary Abs specific for actin (I-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Primary Abs against pSTAT6 were diluted 1/1000 in 5.0% BSA (Sigma-Aldrich, Oakville, Ontario). Primary Abs against STAT6, pSMAD2, and actin were diluted 1/1000 in 5.0% milk. For detection of primary pSTAT6 and STAT6, secondary goat anti-mouse IgG HRP Ab was purchased from Santa Cruz Biotechnology. Goat anti-mouse IgG HRP Ab was diluted 1/2500 in 5.0% milk. For detection of primary pSMAD2, secondary goat anti-rabbit IgG HRP Ab was purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgG HRP Ab was diluted 1/2500 in 5.0% milk. For detection of primary actin (I-19) Ab, rabbit anti-goat IgG HRP Ab was purchased from Sigma-Aldrich. Rabbit anti-goat IgG HRP Ab was diluted 1/2500 in 5.0% milk.

Whole mouse lung tissue was homogenized in radioimmunoprecipitation (RIPA) lysis buffer (1x PBS (pH 7.4), 1.0% igepal CA-630, 0.5% sodium deoxycholate ($C_{24}H_{39}NaO_4$), 0.1% SDS, including inhibitors (1 mM PMSF, 1 mM sodium

orthovanadate (Na_3VO_4), and 1 mg/ml aprotinin). Lung homogenates were incubated for 1 hour on ice. Lung homogenates were then vigorously sheared using a syringe with a 21-gauge needle, centrifuged at maximum speed (12,000 x *g*) at 4°C, supernatant collected, and stored at –70°C. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad, Mississauga, Ontario). Equal amounts of protein were loaded onto 8% SDS-PAGE gels for electrophoresis. Proteins were transferred to Immobilon-NC membranes (Millipore, Ottawa, Ontario). Immunoblots were blocked in 1x TBS containing 0.15% Tween 20 and 5.0% fat-free milk powder for 1 h at room temperature and probed with primary Abs overnight at 4°C. Membranes were washed, incubated with HRP-conjugated secondary Abs for 1 hour at room temperature, and visualized using an ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec).

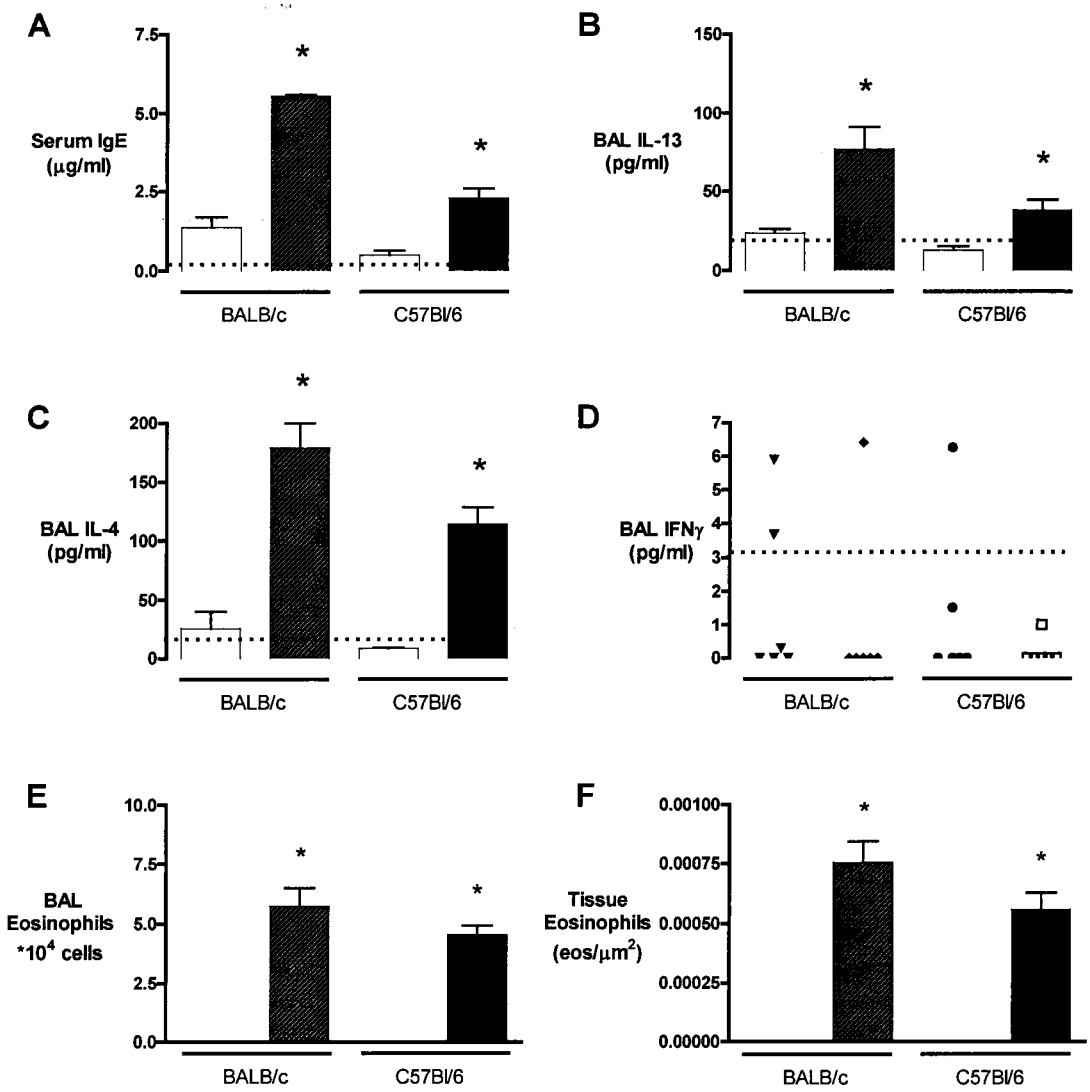
Immunoblots were exposed by X-OMAT Kodak Scientific Films and images scanned at 300–400 dpi resolution. Band density was analyzed for pSTAT6, STAT6, pSMAD2 and loading control (actin) using BioRad Quantity One software. Density levels for pSTAT6, STAT6, and pSMAD2 were normalized using density values of the loading control (actin) and fold induction for these proteins was quantified.

Gene Expression Analysis: Gene expression analysis was performed 2wks following a single period of allergen exposure (Day 34). Following euthanasia, lungs were removed, rinsed in PBS, and immediately immersed in RNAlater (Ambion, Streetsville, ON, Canada) and stored at 4°C elsius overnight as per the manufacturer's recommendations.

RNA isolation was performed using a Qiagen MiniPrepKit (Mississauga, ON, Canada) and concentration and purity were assessed by optical density measurements. RNA was transcribed using a standard protocol (Invitrogen Life Technologies, Burlington, ON). Quantitative real-time PCR was conducted using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). Negative control samples (no template or no reverse transcriptase) were run concurrently. Relative quantification was performed (ddCT method) with results normalized to GAPDH and all values expressed as a fold increase over a naïve C57Bl/6 whole lung homogenate. Assay on Demand products for TGF-β1 and GAPDH were purchased from Applied Biosystems.

Lung Deposition PET/CT study – Mouse Strain Comparison: Animals were anaesthetized using isoflurane. Approximately 200 uCi of 18-FDG in 25 μl of PBS was administered intranasally (IN) using the same technique used to deliver the allergen. Immediately after IN delivery, a whole body static scan using a small animal positron emission tomography (PET, Mosaic PET, Philips Healthcare) was acquired followed by a computed tomography (CT). The PET and CT images were fused to determine the anatomical location of the radioactivity. Radioactivity counts were adjusted for amount of activity administered and body weight of the animal. Regions of interest were drawn over the lung, nose, and whole body and radioactivity in the areas were deteremined.

Results:



Supplement Figure 1: Inflammatory profiles of mice following brief allergen exposure. **A** – Serum IgE levels determined by ELISA (level of detection 1.6 ng/ml). **B** – BAL supernatant IL-13 levels (level of detection 19.5 pg/ml). **C** – BAL supernatant IL-4 levels (level of detection 7.8 pg/ml). **D** – BAL supernatant IFN-γ levels (level of detection 3.1 pg/ml). Individual data points are presented as the large majority of

samples were below manufacturer recommended level of detection and a bar graph could not be generated. **E** – BAL eosinophil counts (*10^4 cells). **F** – Tissue eosinophil counts (eosinophils/μm^2).

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

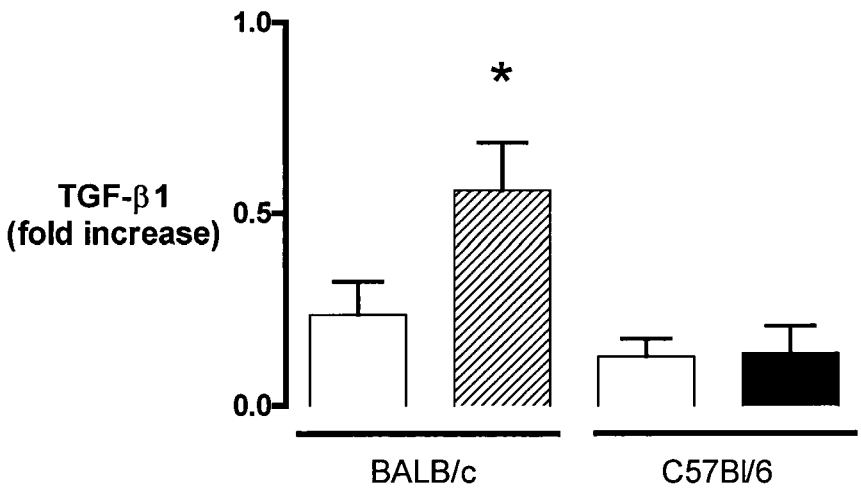
C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 10 mice per group.

Dashed lines represent level of detection of ELISA kit

* significantly different from corresponding control animals ($p<0.05$)



Supplement Figure 2: TGF-β1 gene expression in lungs from BALB/c and C57Bl/6 mice following brief allergen exposure. Gene expression was performed 2wks following a single 2-day period of allergen exposure (Day 34) in BALB/c mice and C57Bl/6 mice.

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 8 mice per group.

CHAPTER 4

AN ESSENTIAL ROLE FOR A NOVEL EPITHELIAL GABAERGIC SYSTEM IN MUCUS OVERPRODUCTION IN ASTHMA

Xiang, Y.Y., Wang, S., Liu, M., **Hirota, J.A.,** Li, J., Ju, W., Fan, Y., Kelly, M.M., Ye, B., Orser, B., O'Byrne, P.M., Inman, M.D., Yang, X., and Lu, W.Y.

The following study is published in:
Nature Medicine. **13**: 862-7 (2007)

Jeremy Hirota's contributions:

As an accessory author I had **NO INPUT** into the study planning, execution, data analysis, figure generation, manuscript preparation in the **pre-submission stages.** During the manuscript **revision** process M.D. Inman and myself were consulted to perform a brief allergen exposure intervention study and provide sliced archived tissues for immunohistochemical staining. My contributions were limited to completing the intervention study, analyzing the airway physiology data, inflammatory cell counts, goblet cell numbers, and generation of **FIGURE 4F and providing tissue slices for supplementary FIGURE 6.**

Reasons for including this paper in my thesis have been outlined in the **Introduction : Relation of publications to general theme of thesis.**

An essential role for a novel airway epithelial GABAergic system in mucus overproduction in asthma

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Abstract: Gamma-aminobutyric acid (GABA) is a major neurotransmitter, which, via the subtype A GABA receptors ($GABA_AR$), induces inhibitions in the adult brain. Here, we report that an excitatory, rather than inhibitory, GABAergic system exists in airway epithelial cells (ECs). Both $GABA_ARs$ and the GABA synthetic enzyme glutamic acid decarboxylase (GAD) are expressed in pulmonary ECs. Activation of $GABA_ARs$ induces a depolarization in these cells. The expression of GAD in the cytosol and $GABA_ARs$ in the apical membranes of airway ECs increases dramatically when mice are sensitized and then challenged with ovalbumin (OVA), an approach for inducing allergic asthmatic reactions. Similarly, GAD and $GABA_ARs$ in airway ECs of human asthmatic subjects increase following allergen inhalation challenge. Importantly, intranasal application of selective $GABA_AR$ inhibitors suppresses the OVA-induced or interleukin-13 (IL-13)-induced hyperplasia of goblet cells and mucus overproduction in mice. These findings demonstrate the essential role of a novel epithelial GABAergic system in asthma.

Airway ECs play an integral role in airway function and disease[1]. Recent reports showed that subunits of $GABA_AR$ are expressed in the type II alveolar ECs (type II ECs)[2]. However, it is not known whether airway ECs also express $GABA_ARs$. Considering the essential role of GABAergic transmissions in brain function[3] and neural diseases[4], we sought to determine if airway ECs possess a functional GABAergic signaling system and, more importantly, whether this system plays a role in airway physiology or pathophysiology.

We first performed immunoblot assays to examine the expression of $GABA_AR$ subunits and GAD65 and 67 (GAD65/67) in pulmonary ECs. The results showed that GAD65/67 and several types of $GABA_AR$ subunits were distinctively expressed in the human bronchial EC line (BEAS-2B cells) and human alveolar type II EC line (A549 cells), as well as in primary human small airway ECs (SAEC) and mouse lung tissues (Fig. 1a). Notably, the expression profiles of GAD and $GABA_ARs$ in BEAS-2B cells were similar to that in mouse lung tissues. In line with the immunoblotting results, reverse transcription polymerase chain reaction (RT-PCR) assays detected the mRNAs encoding GAD67 and α2, β2 and π subunits of $GABA_ARs$ in BEAS-2B cells (Supplementary Fig. 1 online). These results suggested that GABAergic signaling-related molecules are expressed in airway ECs. To investigate the cellular localization of the GABAergic system in the lung, we carried out immunoflourescent staining of GAD65/67 and $GABA_ARs$ in the naïve mouse lung slices. Since immunoblot revealed a higher level of the $GABA_AR$ β2 subunit in the mouse lung (Fig. 1a), and since in neurons β2- or β3-subunits are required for most of functional $GABA_ARs$[5], we stained $GABA_ARs$ using an antibody recognizing both the β2 and β3 subunits. Confocal microscopy of the stained tissues revealed that GAD65/67 was expressed in all ECs in the bronchial airway (Fig. 1b), but in only a small proportion of the alveolar ECs (Fig. 1b, inset). The $GABA_AR$ β2 or β3 subunit was stained on the apical membrane of a small proportion of airway ECs (Fig. 1c), and certain alveolar ECs (Fig. 1c, inset). These results showed that under normal conditions, $GABA_ARs$ and the GABA-production mechanism are indeed present,

albeit at low levels, in bronchial airway ECs, thus forming a complete GABAergic system.

$GABA_AR$s are pentameric Cl^- channels. We performed perforated patch recordings in widely-used primary human SAECs[6] and primary human type II ECs[7]. Under voltage-clamp mode at a holding potential of –60 mV, application of GABA (100 μmol/L) evoked rapid inward current in 4 of 26 tested SAECs (Fig. 1d, left), whereas 4 of 4 tested type II ECs generated inward currents in response to GABA (data not shown). Under current-clamp mode, GABA induced a membrane depolarization in these cells (Fig. 1d, right). The GABA-evoked currents were effectively blocked by the $GABA_AR$ antagonists picrotoxin (50 μmol/L) (Fig. 1e), or bicuculline (100 μmol/L) (Supplementary Fig. 2 online and Fig. 1e, right). Further studies revealed that the reversal potential of GABA-currents (E_{GABA}) in A549 cells (Fig. 1f) was –12 ± 2.4 mV (n = 7), and the "resting" potential (E_R) of these cells was –43 ± 2.6 mV (n = 9) (Fig. 1f, right). These analyses predict that $GABA_AR$s in lung ECs mediate an anionic efflux. Moreover, application of picrotoxin to A549 cells generated an outward current under voltage-clamp mode (Fig. 1g left, n = 6) and induced hyperpolarization under current-clamp conditions (Fig. 1g, right, n = 5). These results suggested that the autocrine or paracrine GABA system persistently maintains pulmonary ECs in a depolarized state.

We then studied whether the airway epithelial GABAergic system plays a role in physiological or pathological processes. It has been well established that Cl^- transport in airway ECs crucially regulates the cell proliferation and mucus production. Using the

bromdeoxyuridine (BrdU) cell proliferation assay, we found that treating the cultured primary SAECs with GABA increased the BrdU incorporation, while addition of picrotoxin reduced the action of GABA (Fig. 1h), In addition, treating SAECs with GABA increased both intracellular and extracellular stains of alcian blue (Supplementary Fig. 3 online), which implied an increased production and secretion of mucin-like glycoprotein[8]. These results imply that $GABA_AR$ activation enhances the proliferation of airway ECs and increases the production of mucus in the airway epithelium.

Airway goblet cell hyperplasia and mucus overproduction are prominent pathological changes of the exacerbation of asthma[9]. To investigate whether the GABA signaling contributes to the pathogenesis of asthma, we sensitized and then challenged mice with OVA, a widely-used approach for inducing allergic asthma-like reactions in animals[10]. Remarkably, the expression of GAD in the cytosol (Fig. 2a,c) and $GABA_AR$ subunits in the apical membrane of mouse airway ECs (Fig. 2b,d) increased significantly following the allergen challenge. Immunoblots of the mouse lung tissues confirmed an increase in the expression of GAD and $GABA_ARs$ (Supplementary Fig. 4 online). Similarly, immunohistochemistry of human biopsies revealed that the expression of GAD in the cytosol (Fig. 2e,f, $n = 6$) and $GABA_AR$ subunits in the apical membrane (Fig. 2g) of the airway ECs increased significantly in the tissues taken from asthmatic subjects 24 h after allergen inhalation challenge, in comparison to the samples collected from the same subject before the allergen challenge. These results indicate that the GABAergic system in the airway ECs is indeed up-regulated during asthmatic reactions. Of note, the $GABA_AR$ subunits were not stained in the smooth muscles of airways in naïve or OVA-

treated mice (Supplementary Fig. 5 online), nor in human airway smooth muscle cells (not shown), which implied that the GABA signaling is selectively associated with epithelial cells.

We then determined the factor(s) that mediate the allergen-induced increase of GABA signaling molecules in airway ECs. IL-13, a classical T_H2 cytokine, could be one of the factors, because it is a key regulator of allergic asthma[11,12]. Using previously described assays[13], we confirmed that the levels of IL-13 in the bronchoalveolar lavage (BAL) and the production of IL-13 by lymphocytes were elevated in OVA-treated mice (Fig. 3a). Treating the cultured SAECs with IL-13 increased the expression of GAD (Fig. 3b) and $GABA_AR$ β2 subunits (Fig. 3c). In addition, 8 out of 12 (67%) cells in the IL-13-treated dishes exhibited currents (9.2 ± 3.0 pA, n = 8) in response to application of GABA, whereas only 2 out of 10 (20%) cells in control dishes did so. In line with these *in vitro* results, intranasal (i.n.) application of IL-13 to mice significantly enhanced the expression of GAD (Fig. 3d,e) and $GABA_AR$ β2 and β3 subunits (Fig. 3f,g) in bronchial ECs. Furthermore, challenging the mice that lack the IL-13 gene (IL-13KO) did not affect the expression of GAD and $GABA_ARs$ in airway ECs (Supplementary Fig. 6 online). These data indicate that IL-13 plays a crucial role in initiating the airway epithelial GABA signaling in allergic asthma.

To confirm the causal relationship between the epithelial GABA signaling and the hyperplasia of goblet cells in asthmatic reactions, the OVA-sensitized/challenged mice were treated with picrotoxin or bicuculline via intraperitoneal (i.p.) or i.n. application.

Blocking GABA signaling lessened the airway epithelium swelling (Fig. 4a,b) and goblet cells hyperplasia (Fig. 4c). Importantly, the OVA-induced mucus overproduction by airway ECs was significantly reduced by picrotoxin (Figs. 4b,c,d), or by bicuculline (Fig. 4d). The IL-13-induced mucus overproduction was also suppressed by i.n picrotoxin (Fig. 4e). However, blocking $GABA_AR$ failed to affect the OVA-induced increase of IL-13 in the lung (Fig. 4f). Moreover, i.n. bicuculline had no effect on the OVA-induced airway hyperreactivity (AHR) (Fig. 4g). In addition, i.n. or i.p. $GABA_AR$ inhibitors failed to block the OVA-induced inflammatory cell infiltrations in the sub-epithelial interstitial tissue of the airway wall (Fig. 4a inset) or in BAL (Supplementary Fig. 7 online). Considering that IL-13 is produced primarily by T_H2 cells after allergen challenge, we propose that up-regulation of the epithelial GABAergic system is down-stream of IL-13 receptor activation, and that this GABAergic system plays a selective role in goblet cell metaplasia and mucus overproduction.

This study demonstrates that a novel autocrine/paracrine GABAergic system in the airway ECs plays an essential role in the process of goblet cell hyperplasia and mucus overproduction. In animal asthmatic models and human asthmatic subjects, the expression of GABAergic signaling molecules was up-regulated following allergen challenge, demonstrating a true relevance of this GABAergic system in asthma. IL-13 plays a critical role in regulating the airway epithelial GABA signaling. In this regard, previous studies have shown that cytokines enhance the expression of $GABA_ARs$ in neurons[14], and $GABA_AR$ activation induces neural progenitor differentiation[15].

Mucus overproduction is the primary cause of death in severe asthma attacks[16,17]. Since the currently available treatments for mucus hypersecretion have limited efficacy and are largely non-specific[9], an effective and specific treatment for mucus overproduction is urgently needed. The airway apical-membrane-located $GABA_AR$ may serve as an easily accessible target for therapeutic reagents. Indeed, intranasal administration of $GABA_ARs$ antagonists reduced the allergen-induced airway mucus overproduction in the mouse model of asthma. Our data not only demonstrate an essential role for a novel epithelial GABAergic system in airway mucus production, but may also lead to new therapeutic strategies for the management of severe asthma.

Methods

Mouse models of allergic asthmatic reactions: Allergic asthmatic reactions were induced in mice using two methods. With the first method as previously described[13], female BALB/c mice (6 to 8 weeks old, from Charles River Laboratories) were initially sensitized with 2 μg OVA (ICN Biomedicals) in 2 mg $Al(OH)_3$, via i.p. injection. Two weeks after sensitization, the mice were challenged with 50 μg of OVA (40 μL i.n.). With the second method, recombinant IL-13 (purchased from eBioscience) was administered via i.n. application to female BALB/c mice at 0.5 μg/40μL, on the 1st, 3rd and 5th d. Starting from the day of intranasal OVA challenge, or intranasal administration of IL-13, mice in one group were treated daily with picrotoxin (PTXN, i.p., 0.2 μg/g body weight in 200 μL, or i.n., 0.2 μg/g body weight in 50 μL), or by bicuculline (i.n. 2 μg/g body weight in 40 μL). Mice were sacrificed at day 6 after the OVA challenge, or the IL-13 treatment. All mice used in this study were housed in the Central Animal Care Facility of

the University of Manitoba, and the experimental protocols were approved by the Animal Use Committee of the University of Manitoba.

Mice assessed for the development of AHR subjected to a model of OVA challenge, where the total respiratory system resistance (RRS) is a prominent outcome. Female BALB/c mice, aged 10 to 12 weeks, were purchased from Harlan Sprague Dawley. All mice were housed in environmentally controlled, specific pathogen-free conditions for a one-week acclimatization period and throughout the duration of the studies. All procedures were approved by the Animal Research Ethics Board at McMaster University, and conformed to the NIH guidelines for experimental use of animals. Mice were sensitized as previously described[18]. Briefly, all mice received i.p. injections of OVA conjugated to Al $(OH)_3$ on Days 1 and 11, and i.n. OVA on Day 11. At day 29 and 30, the sensitized mice were subjected to i.n administration of OVA (100 μg in 25 μL saline). In a subgroup of these OVA-challenged mice, bicuculline (2 μg/g body weight in 40 μL) was given i.n. RRS responses to intravenous saline and increasing doses of methacholine (MCh) were performed at the 24th h after the second OVA challenge using the FlexiVent ventilator system (SCIREQ)[18].

BAL analyses: As previously described[19], the trachea of each mouse was cannulated after euthanasia, and the lungs were washed twice with 1 mL phosphate buffer solution (PBS). Cells in the fluid samples were counted, and the samples were then spun down. The pellets were re-suspended with saline, and slides were prepared for differential cell counting. The cells on the slides were stained with Fisher Leukostat Stain Kit (Fisher Scientific). The numbers of monocytes, lymphocytes and eosinophils (identified by

morphology and staining characteristics) in a total of 200 cells on each slide were counted. The level of IL-13 in the BAL fluid samples was measured using enzyme linked immunosorbent assay (ELISA) as previously described[13].

Western blotting: Cultured lung ECs and mouse lung tissues were lysed in ice-cold PBS with 1% Triton X-100 and 0.5% sodium deoxycholate supplemented with protease inhibitors. The general procedures of Western blotting were the same as previously described[20]. The antibodies to GAD 65/67, $GABA_AR$-α5 and ß-actin were purchased from Sigma. The antibody to $GABA_AR$-α2 was from Alomone Labs. The antibodies to $GABA_AR$-β1 and β3 were from Affinity Bioreagents. The antibodies to $GABA_AR$-β2 and -δ were from Chemicon, and the antibody to $GABA_AR$-π was from Abcam. For quantification, the blotting films were scanned by means of a GS800 densitometer (Bio-Rad), and the band densities were calculated using the Quantity One program (Bio-Rad). The blotting assays were repeated at least 3 times with lung tissue samples from 3 mice. For blotting assays of GAD and most $GABA_AR$ subunits, the mouse cerebral cortex was used as the positive control. For the π subunit, the Jacket cell lysate was used as the positive control.

Human airway biopsies: Airway biopsies were obtained from six subjects with mild asthma and using no medication other than infrequent (< 5 times weekly) inhaled β2-agonists to treat their symptoms. The subjects had not had an asthma exacerbation or a respiratory tract infection for at least 4 weeks before the study. The diagnosis of asthma was based on the presence of variable airflow limitation and airway

hyperresponsiveness[21]. All subjects were nonsmokers and demonstrated an allergen-induced early and late asthmatic response[22]. These subjects underwent sequential diluent (control) and allergens inhalation challenges as described previously[23,24], separated by a period of at least 3 weeks. Fibreoptic bronchoscopy and endobronchial biopsy was performed according to the recommendations of the U.S. National Institutes of Health[25], 24 h after challenge. Mucosal biopsies were taken from the segmental and subsegmental carinae of the lung and fixed in 10% buffered formalin for 24 h. The study of allergen induced airway responses in mild asthmatic subjects was reviewed and approved by the Human Research Ethics Board of McMaster University before the study began and all subjects gave informed consent before being enrolled into the study.

Immunohistochemistry and confocal microscopy: Paraffin sections of mouse lung tissue and human bronchial airway biopsy were deparaffinized with xylene and then dehydrated in 100%, 95%, and 70% ethanol. Epitopes were unmasked by heating the tissue sections in citrate buffer at pH 6 in a microwave. The tissues were permeabilized with 0.1% Triton X-100 and blocked with 10% normal goat serum for 1 h. The slices were incubated overnight with primary antibodies (antibody to GAD 65/67, 1:800 dilution; antibody to $GABA_AR$ β2 and β3, 1:100 dilution; Upstate; antibody to MUC5AC/clone 45M1, 2 μg/ml; Lab Vision Corp; antibody to α smooth muscle actin, 1:1,000 dilution; Abcam), and subsequently with CY3-conjugated or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. An FITC-conjugated antibody to pan-cytokeratin antibody (1:100 dilution; Sigma) was used to visualize ECs in the lung. When mouse monoclonal antibodies were used on mouse sections, immunofleorescence

was performed using mouse on mouse (M.O.M.) kit (Vector Laboratories). Controls were performed either without primary antibodies or incubated in mouse IgG (Santa Cruz Biotechnology) to ensure stain specificity. The immunohistochemistry of each protein was repeated in lung tissue slices of 3 to 6 mice. Immunocytochemistry of cultured cells was performed as previously described[20,26]. Confocal images of stained lung tissue or lung ECs were studied via an inverted microscope (Carl Zeiss) using the Zeiss LSM program. The fluorescence intensity for a specific protein stain was set below the threshold for the negative control. Digital images of lung tissues containing small airways and/or alveolar structures were obtained for analysis.

H & E staining and mucus analysis: Lung tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained by hematoxylin and eosin (H & E) and examined for pathological changes under light microscopy. Mucus and mucus-containing goblet cells within the bronchial epithelium were stained with a periodic acid-Schiff (PAS) staining kit (Sigma). The histological mucus index (HMI), the percentage of mucus-positive area of the whole bronchial epithelium[13], was determined by Image-Pro Plus software (Media Cybernetics).

Cell culture: Lung ECs were plated in culture dishes (Nunc) or on glass coverslips and incubated at 37°C in a humidified atmosphere of 5% CO_2. Isolated primary human type II ECs[27], BEAS-2B cells and A549 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. The primary human SAECs were purchased from Cambrex Bio Science Walkersville, Inc., and were cultured in

small-airway growth media (SAGM, Cambrex Bio Science Walkersville, Inc.) on dishes coated with collagen I (BD Biosciences). Procedures of culturing lymphocytes from spleen and draining lymph nodes of OVA-treated mice and analyzing allergen-driven IL-13 production by these cells were performed as previously described[13,28].

BrdU Assay: The SAEC proliferation was quantified by measuring the ability of cells to incorporate bromdeoxyuridine (BrdU) using ELISA. For the assay, SAEC were seeded on to 96-well plates at a density of $3x10^3$/well. Twenty four hours after seeding, triplicate wells were treated with GABA (10 μmol/L), picrotoxin (PTXN, 25 μmol/L), or GABA plus PTXN for 24 h. BrdU (10 μmol/L) was added in the cultures 6h before assay, which was performed in accordance with the manufacture's instructions provided with the BrdU ELISA Kit (Roche Applied Science).

Electrophysiology: After removal of the culture media, lung ECs were bathed in a solution that contained (in mmol/L): 155 NaCl, 1.3 $CaCl_2$, 5.4 KCl, 25 HEPES, and 33 glucose, at pH 7.4 and osmolarity about 315 mOsm. An Axopatch-1D amplifier (Axon Instruments) was used to make perforated patch recordings at room temperature. The patch electrodes were filled with a solution that contained (in mmol/L) 155 KCl, 15 KOH, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, and 2 tetraethylammonium, at pH 7.35 and osmolarity 315 mOsm. Gramicidin (15–20 μg/mL)[29] was included in the electrode solution for membrane perforation. Application of the $GABA_AR$ agonist and/or antagonist was achieved via a computer-controlled multibarrel perfusion system (SF-77B, Warner Instruments). Electrical signals were digitized and filtered (1–2 kHz).

Transmembrane currents were acquired on-line by means of Clampex (Axon Instruments), and the data were analyzed off-line using Clampfit (Axon Instruments).

Statistical Analysis: Statistical analyses were performed with Sigmaplot software (SPSS). Data are expressed as mean ± standard error of the mean (s.e.m.) and were examined with Student's unpaired or paired *t* tests when appropriate. A *p* value less than 0.05 was considered significant.

Acknowledgements

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Figure Legends

Figure 1: An excitatory GABAergic system in lung ECs. **a.** Immunoblot of human pulmonary EC lines (BEAS-2B and A549 cells), primary human small airway ECs (SAEC) and mouse lung for GAD65/67 and $GABA_AR$ subunits (as labeled at the left of each blot). **b** and **c**. Immunohistochemistry reveals the cellular distribution of GABAergic molecules in the mouse lung. Mouse lung ECs were demonstrated by immunostaining with an antibody to pan-cytokeratin (green). Immunostaining of GAD65/67 (red, in **b**); scale bar, 40 μm. Staining of $GABA_AR$ β2/β3 subunits (red, in **c**); Scale bar, 15 μm. **d.** The representative trace of GABA-evoked currents (left panel) and GABA-induced depolarization (right panel) in primary human SAECs. **e.** The GABA-induced current was blocked by picrotoxin (PTXN) (left panel). Summary of effects of PTXN and bicuculline (Bicu) on GABA-evoked currents (right panel, in comparison to control, *** $P < 0.001$; ** $P < 0.01$). **f.** Representative traces of GABA-currents under different holding membrane potentials (from –60 to 40 mV, 20mV-step; left panel); and summary of the endogenous resting membrane potential (E_R) and the reversal potential of GABA-induced current ($E_{GABA)}$ (right panel). **g.** PTXN induced outward current (left panel) and hyperpolarization (right panel). These data indicate a "tonic" GABAergic activity in the pulmonary ECs. **h.** ELISA results of the effect of GABA on BrdU incorporation to SAEC. The unit of BrdU was a value of absorbance normalized to the control reading. * in comparison to all other groups, $P < 0.05$.

Figure 2: OVA-treatments increase the expression of airway GABAergic signaling components. **a.** Representative confocal images of immunostaining of GAD (red, middle

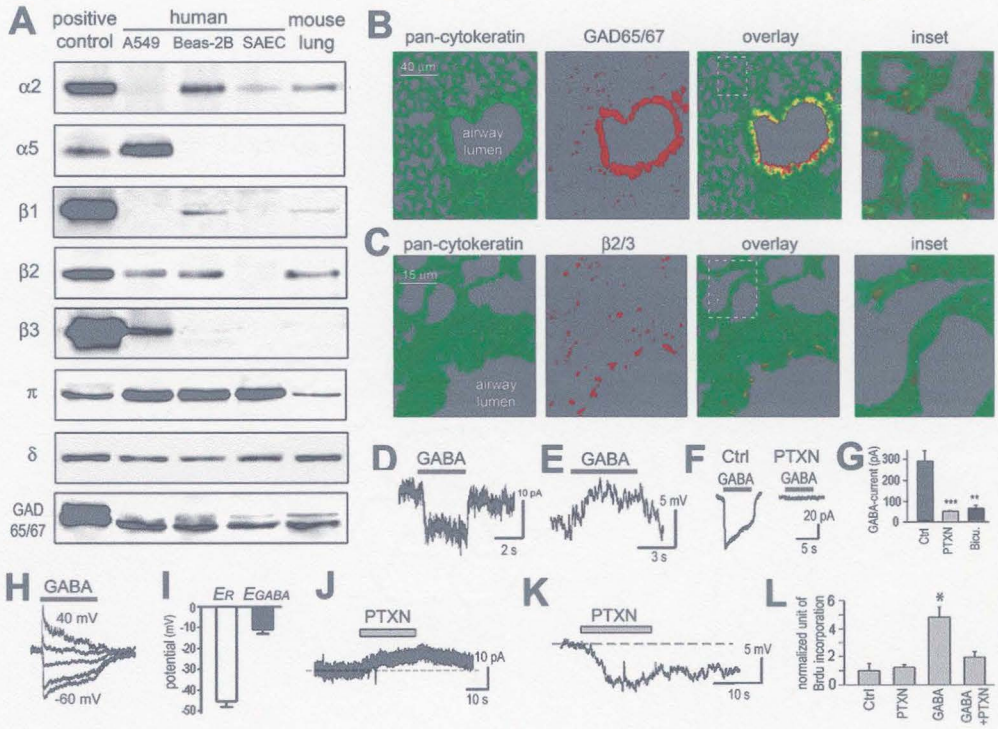
panels) in the lung tissues from control (Ctrl) and OVA-treated (OVA) mice. Scale bar, 40 μm. **b.** Typical confocal images of immunostaining of $GABA_AR$ β2/β3 subunits (red, middle panels) in lung tissues from control (Ctrl) and OVA-treated (OVA) mice. Scale bar, 15 μm. The immunoflourescent staining is semi-quantitatively analyzed, showing as fluorescent pixels per image field. Summaries of the fluorescence density of the GAD (in **c**) and β2/β3 subunit (in **d**) in the airway epithelium of control mice and OVA-treated mice (n = 20 image fields of 8 lung slices from 4 mice; ***, $P < 0.00001$). **e.** Representative confocal images of immunostaining of GAD in airway biopsies from a human asthmatic subject, who was consecutively treated by inhaling diluent (control) and combined allergens (challenge) with 24 h intervals. Scale bar, 20 μm. **f.** Summary of the GAD fluorescence density in the airway epithelium of human biopsies (n = 6 cases; * P <0.01). **g.** Typical confocal images of immunostaining of $GABA_AR$ β2/β3 subunits in airway biopsies from the same subject described in **e**. Scale bar, 20 μm. Insets show the increased expression of $GABA_ARs$ in the apical membrane of airway ECs after allergen inhalation challenge.

Figure 3: Pulmonary IL-13 increases during allergic asthma and stimulates the expression of GAD and $GABA_ARs$ in airway ECs. **a.** ELISA revealed increases of IL-13 in BAL fluid (left), in the culture supernatants of spleen cells (meddle) and draining lymph node cells (right) from the OVA-treated mice. **b.** Representative confocal images of immunostaining of GAD65/67 (green) in control and IL-13 (5 ng/mL for 6 d)-treated SAECs. The nuclei were stained with propidium iodide (red). Fluorescent assay showed

an increase in the intensity of GAD65/67 in the IL-13 treated cells (control 39.9 ± 5.3 pixels/225 μm^2, n = 63 cells; IL-13 86.2 ± 4.9 pixels/225 μm^2, n = 89 cells in 2 experiments; $P< 0.01$). **c**. Immunoblot of β2-subunits in control and IL-13 treated SAECs. **d**. Representative confocal images show immunostaining of GAD65/67 (red) in lung tissues from the control and intranasal (i.n.) IL-13-treated mice. **e**. Summary of the immunofluorescence density of GAD in lung tissues from the control and IL-13-treated mice (* $P < 0.01$). **f**. Typical confocal images show the immunofluorescent staining of $GABA_AR$ β2 and β3 subunits (red) in lung tissues from control and IL-13-treated mice. Scale bar in **d** and **f**, 20 μm. **g**. Summary of the immunofluorescence density of β2 and β3 subunits in lung tissues from the control and IL-13-treated mice (* $P < 0.01$).

Figure 4: GABAergic blockade decreases OVA-induced airway goblet cell hyperplasia and mucus overproduction. **a**. Typical histological images (H & E staining) of lung sections from a control mouse, an OVA-challenged (OVA) mouse, and an OVA-challenged mouse that was treated with i.n. picrotoxin (OVA+PTXN). The insets show the inflammatory cell infiltrations surrounding the airway. **b**. Typical PAS-staining of lung tissues from the control, OVA-treated and OVA+PTXN-treated mice. Scale bar, 60 μm. **c.** Representative images of immunostaining of mucin (green) with an antibody to MUC5AC. **d**. Summary of histological mucus index (HMI) (which indicates the proportion of mucus-producing epithelium in the bronchial epithelium) obtained from the OVA-treated and OVA+inhibitor-treated mice. Note that i.p. or i.n. application of PTXN, or bicuculline (Bicu) significantly reduced mucus production (** $P < 0.001$, in comparison to OVA-challenged mice; n = 3 mice). **e**. Summary of HMI from the IL-13-

treated and IL-13+PTXN-treated mouse lung tissues (* $P < 0.05$, $n = 3$ mice). **f.** Summary of ELISA of IL-13 in BAL from mice in different testing groups. **g.** Effect of intranasal application of the $GABA_AR$ inhibitor, bicuculline, on OVA-induced AHR. Note that inhibition of $GABA_AR$ did not affect AHR.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positive control
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A549
Beas-2B
SAEC
mouse lung
α2
α5
β1
β2
β3
π
δ
GAD 65/67
B
pan-cytokeratin
GAD65/67
overlay
inset
40 µm
airway lumen
C
pan-cytokeratin
β2/3
overlay
inset
15 µm
airway lumen
D
GABA
10 pA
2 s
E
GABA
5 mV
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F
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PTXN
GABA
GABA
20 pA
5 s
G
GABA-current (pA)
300
200
100
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Ctrl
PTXN
Bicu.
H
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40 mV
-60 mV
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potential (mV)
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-10
-20
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-40
-50
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5 mV
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normalized unit of Brdu incorporation
6
4
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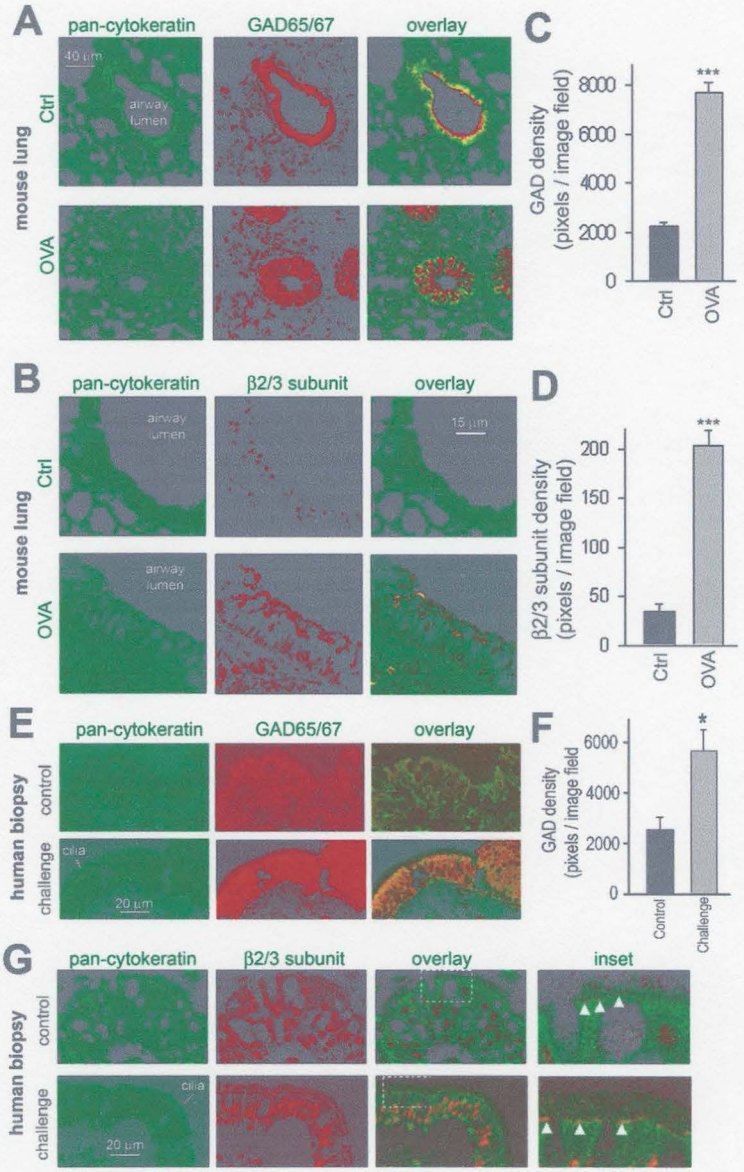
PTXN
GABA
GABA +PTXN

Figure 1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OVA
40 µm
airway lumen
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control
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GAD density (pixels / image field
6000
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Control
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G
pan-cytokeratin
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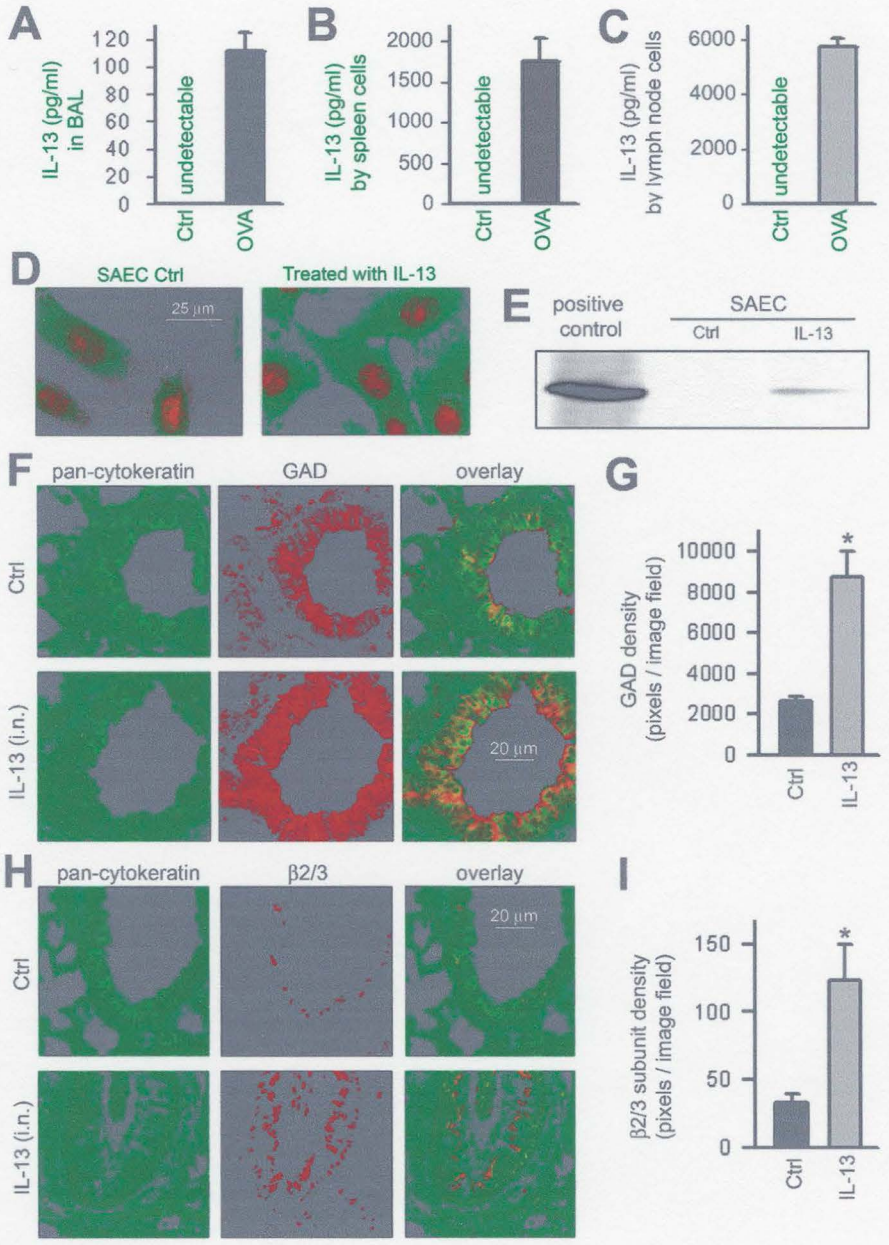
human biopsy
control
challenge
cilia
20 µm

Figure 2


A
IL-13 (pg/ml) in BAL
120
100
80
60
40
20
0
undetectable
Ctrl
OVA
B
IL-13 (pg/ml) by spleen cells
2000
1500
1000
500
0
undetectable
Ctrl
OVA
C
IL-13 (pg/ml) by lymph node cells
6000
4000
2000
0
undetectable
Ctrl
OVA
D
SAEC Ctrl
Treated with IL-13
25 µm
E
positive control
SAEC
Ctrl
IL-13
F
pan-cytokeratin
GAD
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Ctrl
IL-13 (i.n.)
20 µm
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GAD density (pixels / image field)
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Figure 3

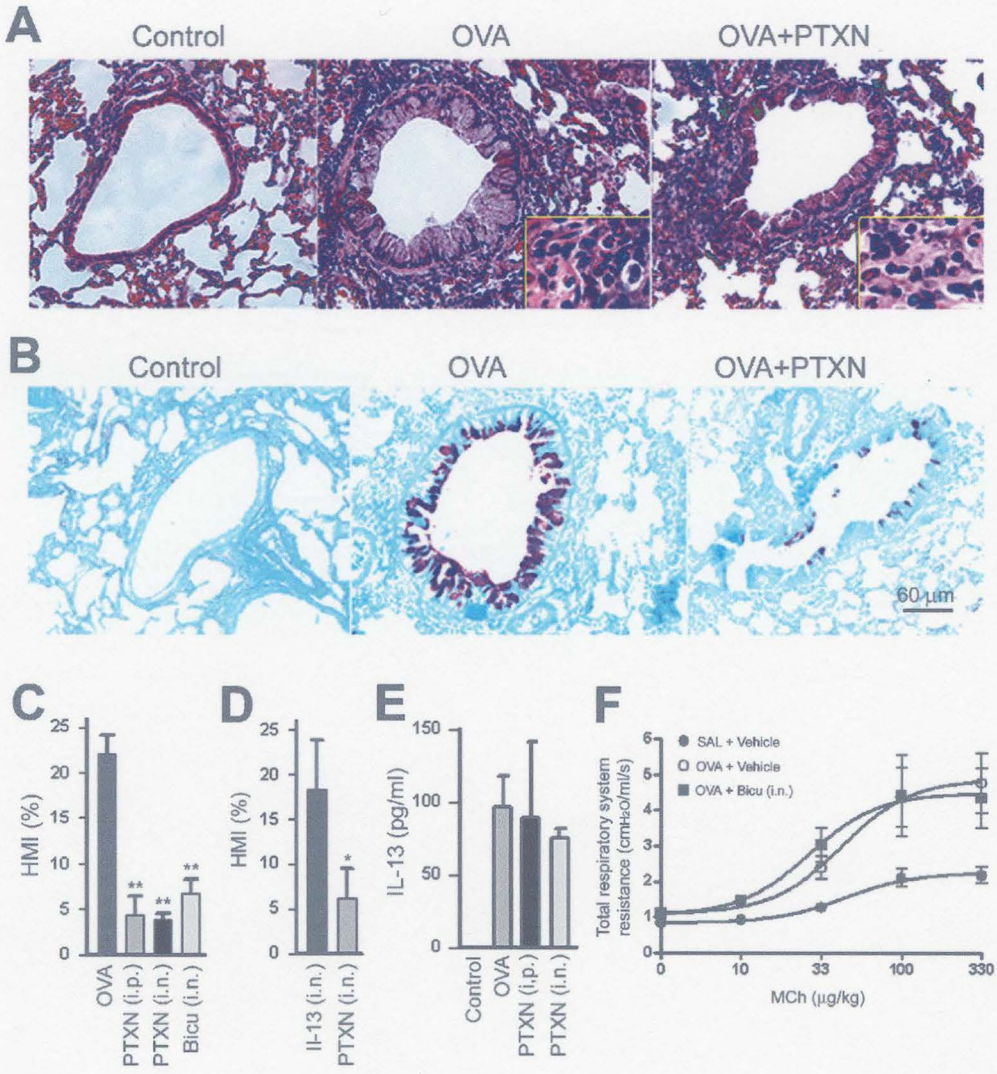

A
Control
OVA
OVA+PTXN
B
Control
OVA
OVA+PTXN
60 µm
C
HMI (%)
OVA
PTXN (i.p.)
PTXN (i.n.)
Bicu (i.n.)
D
HMI (%)
Il-13 (i.n.)
PTXN (i.n.)
E
IL-13 (pg/ml)
Control
OVA
PTXN (i.p.)
PTXN (i.n.)
F
SAL + Vehicle
OVA + Vehicle
OVA + Bicu (i.n.)
Total respiratory system resistance (cmH2O/ml/s)
MCh (µg/kg)

Figure 4

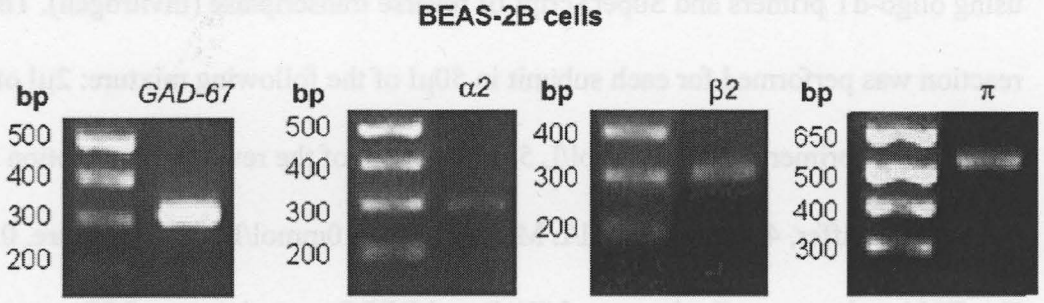
Supplement –Methods and Figures

RT-PCR: The total RNA was isolated from BEAS-2B cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcripts were prepared using oligo-dT primers and SuperScript III reverse transcriptase (Invitrogen). The PCR reaction was performed for each subunit in 50μl of the following mixture: 2μl of 50pmol/L 3' primer, 2μl of 50pmol/L 5' primer, 4μl of the reverse transcription product, 5μl of 10X buffer, 4 μl of 50mmol/L $MgCl_2$, 1μl of 10mmol/L dNTP mixture, 0.5μl of *Taq* DNA polymerase (Invitrogen, 5 U/μl) and DEPC-treated water. PCR was carried out for 30 cycles, each consisting of 30s at 94°C, 30s at 55°C, and 60s at 72°C. The gene specific primers are described in the table below.

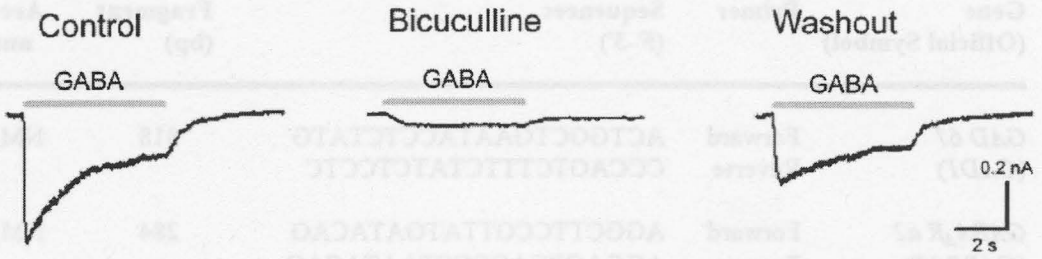
Gene (Official Symbol)	Primer	Sequences (5'-3')	Fragment (bp)	Accession number
GAD 67 (*GAD1*)	Forward Reverse	ACTGGCTGAATACCTCTATG CCCAGTCTTTCTATCTCCTC	318	NM_000817
$GABA_AR\ \alpha 2$ (*GABRA2*)	Forward Reverse	AGGCTTCCGTTATGATACAG AGGACTGACCCCTAATACAG	284	NM_000807
$GABA_AR\ \beta 2$ (*GABRB2*)	Forward Reverse	GCATCCAGTATCGGAAAGCT TCCAAGTCCTACATCAGGCT	300	NM_021911
$GABA_AR\ \pi$ (*GABRP*)	Forward Reverse	CGAGGTCGGCAGAAGTGACAAG TCCTGCTGCGATCTGGTGACTAA	591	NM_014211

Alcian blue staining: SAECs were plated on glass coverslips and grown in SAGM. As grouped, cells were treated with GABA (25μmol/L) in the presence or absence of picrotoxin (25μmol/L) for 6d. The cells were rinsed with PBS and fixed in 4% paraformaldehyde for 15min. The cultures were then stained overnight at 4°C with 1%

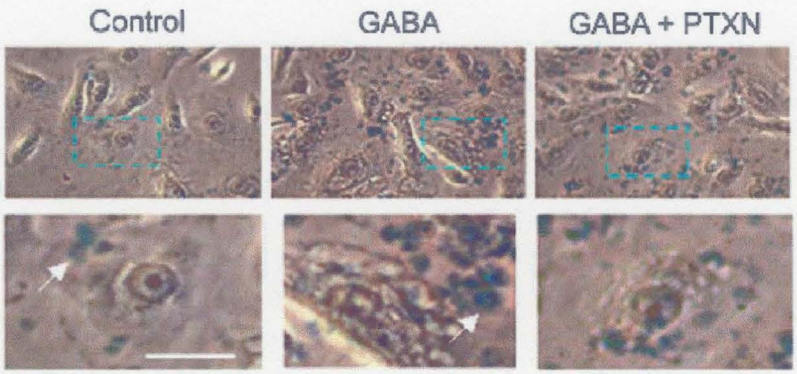
alcian blue 8 GX (Sigma) in 3% acetic acid solution, pH 2.5. After these washes with PBS, the cells were counterstained with 0.1% nuclear Fast Red.



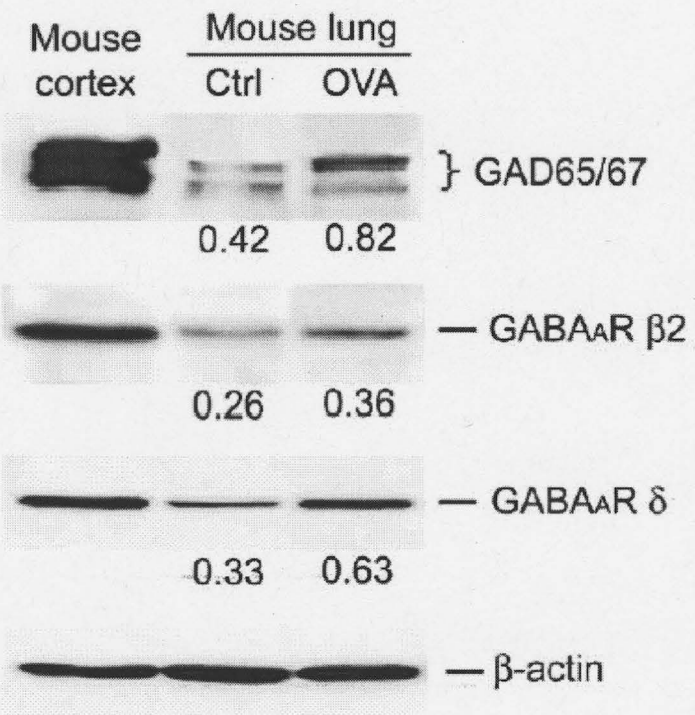
Supplement Figure 1: RT-PCR assays of GAD and $GABA_AR$ subunits in BEAS-2B cells.



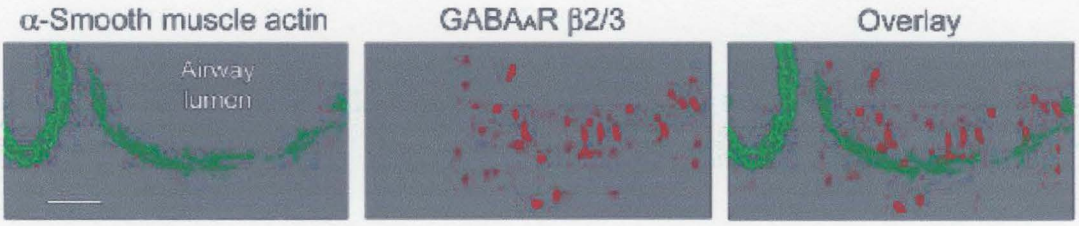
Supplement Figure 2: The selective $GABA_AR$ antagonist bicuculline blocks the GABA-induced current in pulmonary epithelial cells. The current evoked by GABA in A549 cells was blocked by the competitive $GABA_AR$ antagonist bicuculline methobromide (100μmol/L).



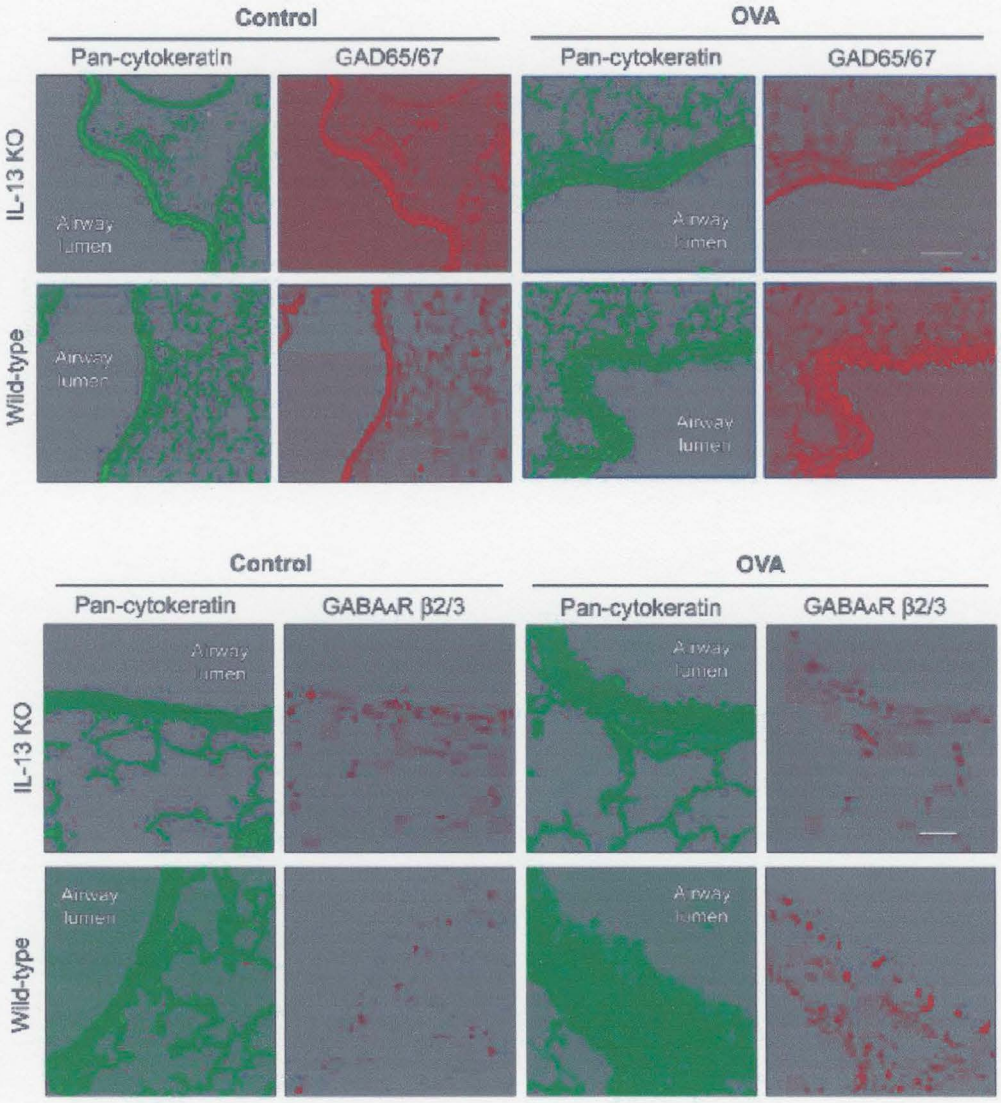
Supplement Figure 3: The intracellular and extracellular alcian blue staining increase in SAEC after GABA treatment. Shown are representative pictures of alcian blue staining (arrow) of SAECs grown under control conditions and treated with GABA or GABA plus PTXN. The enclosed square area in each picture in the upper panel is enlarged and shown in the lower panel. Scale bar, 15μm.



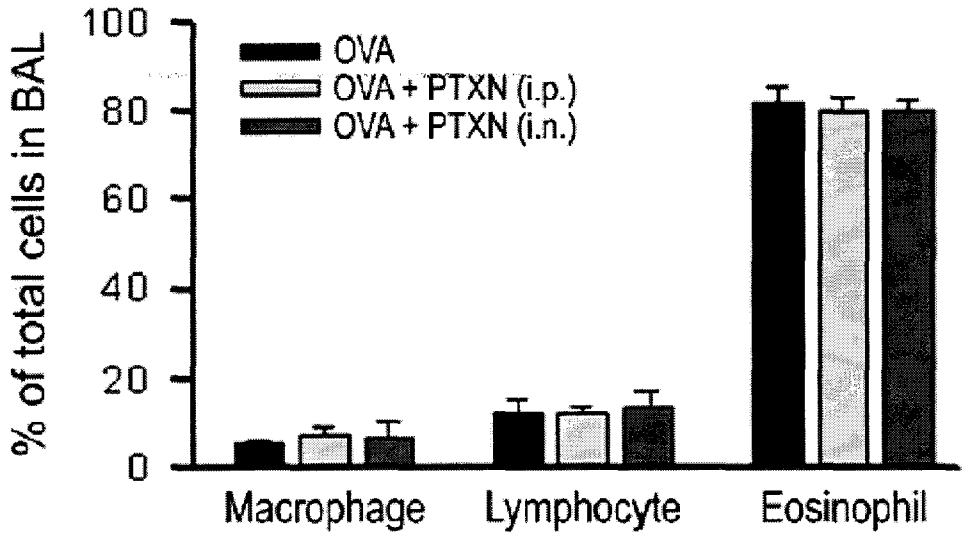
Supplement Figure 4: The expression of GAD and $GABA_AR$ subunits increases in the lung of OVA-treated mice. Immunoblotting assays show expression of GAD65/67 and two $GABA_AR$ subunits in lung tissues from control (ctrl) and OVA-treated mice. The number under each immunoblot band is the density normalized to that of β-actin.



Supplement Figure 5: $GABA_AR$ β2 and β3 subunits are not expressed in the airway smooth muscle cells. Confocal microscopy images show double immunostaining of a lung tissue slice from an OVA-treated mouse, with an antibody against α-smooth muscle actin (green) and the antibody to the β2 and β3 subunits of $GABA_ARs$ (red). Note that no $GABA_AR$ stain is co-localized with smooth muscle staining, indicative of that $GABA_ARs$ are not expressed in airway smooth muscle cells. Scale bar, 30μm.



Supplement Figure 6: The expression levels of GAD and $GABA_AR$s do not increase in the airway epithelial cells in OVA-challenged IL-13 knockout mice. Note: The tissues were taken from wild-type and IL-13 knockout (IL-13KO) mice that were subjected to a previously described procedure of OVA-sensitization/challenge (Leigh,R. et al. *Am.J.Respir.Crit.Car Med.* **170**:851-856.2004). Scale bar in top panel, 100μm; Scale bar in bottom panel, 50μm.



Supplement Figure 7: $GABA_AR$ inhibitor does not affect inflammatory cell infiltration to the lung. Shown are percentage of macrophages, lymphocytes, and eosinophils in BAL fluid from different animal groups.

CHAPTER 5

THE ROLE OF IL-4Rα IN THE INDUCTION OF GLUTAMIC ACID DECARBOXYLASE IN AIRWAY EPITHELIUM FOLLOWING BRIEF HOUSE DUST MITE EXPOSURE

Hirota, J.A., Budelsky, A., Smith, D., Lipsky, B., Ellis, R., Xiang, Y., Lu, W.Y., and Inman, M.D.

The following study is to be submitted to:
Clinical and Experimental Allergy. (June 2009)

Jeremy Hirota's contributions:

As primary author I, J. Hirota, was responsible for conceiving, developing, and managing the entire study. This included tissue collection, processing, immunohistochemistry, and morphometrics. A. Budelsky provided IL-4Rα reagents, dilutions, and dosing protocols. D. Smith provided insight into dosing protocols. B. Lipsky contributed to gene expression analysis. R. Ellis provided insight into figures. Y. Xiang was responsible for confocal microscopy. W.Y. Lu provided reagents and infrastructure for confocal microscopy. Analysis, statistics, figure generation, manuscript preparation, and submission were performed by me with guidance from M.D. Inman. M.D. Inman is the senior author and primary investigator of the lab that the work was completed in.

The role of IL-4Rα in the induction of glutamic acid decarboxylase in airway epithelium following brief house dust mite exposure.

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Key Words :
Asthma, Airway Remodeling, Goblet cells, Mucus, Mouse.

Abstract

Background : Asthma is a disease characterized by airway inflammation, remodeling and dysfunction. Airway inflammation contributes to remodeling, a term that is used to describe structural changes including goblet cell metaplasia (GCM), matrix deposition, and smooth muscle hyperplasia/hypertrophy. GCM has been implicated in asthma mortality by contributing to mucus plugs and leading to asphyxiation. In animal models this process is highly dependent on IL-13. Recently we have described an IL-13 dependent upregulation of a GABAergic signaling system in airway epithelium that contributes to GCM. The mechanism by which IL-13 upregulates GABA signaling in airway epithelium is unknown.

Objectives : Test the hypothesis that IL-4Rα signaling is required for allergen induced upregulation of GABAergic signaling and GCM.

Methods : BALB/c mice were exposed to a brief house dust mite (HDM) protocol and received vehicle, anti-IL-4Rα-monoclonal antibody, or control antibody. Outcomes included airway responses to inhaled methacholine, histology for eosinophilia and GCM, phosphorylated STAT6 levels immunohistochemistry, and glutamic acid decarboxylase (GAD) 65/67 expression using confocal microscopy.

Results : Brief HDM exposure resulted in increased airway responses to methacholine, lung eosinophilia, STAT6 phosphorylation, elevations in GAD65/67 expression, and GCM that were inhibited with anti-IL-4Rα-monoclonal treatment. Control antibody had no effect.

Conclusion : The IL-4Rα is required for allergen induced upregulation of a GABAergic system in airway epithelium implicated in GCM following brief HDM exposure.

Introduction

Asthma is a disease characterized by Th2 skewed airway inflammation, remodeling and hyperresponsiveness. The efficacy of anti-inflammatory treatments in controlling airway function of humans has provided evidence to support the contributing role of inflammation plays in the development of airway dysfunction (1,2). A positive relationship between airway inflammation and remodeling of the airway wall have been observed in animal studies (3,4), although causal links attained from clinical data remain elusive.

Airway remodeling is a broad term that encompasses several changes in the airway wall composition including goblet cell metaplasia (GCM), increased matrix deposition, smooth muscle hyperplasia, and epithelial desquamation(4). The relative contribution of each airway remodeling change to AHR is not clear, although it has been demonstrated that GCM and mucus overproduction contribute to fatal asthma attacks by forming mucus plugs(4). Experimental mouse models of asthma using transgenics, KOs, brief and chronic allergen exposure protocols, have implicated the Th2 cytokine IL-13 in the development of GCM(3,5-7).

IL-4 and IL-13 cytokines share the IL-4Rα chain to form their functionally signaling receptors (8-10). IL-4 is capable of signaling through two distinct receptors that utilize the IL-4Rα chain. The type I IL-4 receptor consists of a common γ chain and the IL-4Rα, while the type II IL-4 receptor consists of the IL-4Rα and the IL-13Rα1. The type II IL-4 receptor is capable of transducing signals for IL-13 in addition to IL-4. In both cases the IL-4Rα binds the cytokine, followed by receptor heterodimerization and

stabilization with the secondary chain (γ chain or IL-13Rα1). The type I and type II IL-4 receptors are constitutively associated with janus activated tyrosine kinases (JAKs) which are responsible for phosphorylating tyrosine residues in the IL4Rα chain to provide docking sites for STAT6. Docking of STAT6 to phosphorylated tyrosine residues on the IL-4Rα results in STAT6 phosphorylation by the same JAKs and subsequent signaling through nuclear import of phosphorylated STAT6 homodimers(8,10,11). Many genes associated with allergy contain STAT6 binding motifs in their upstream promoter regions including eotaxin, IL-4, IL-4Rα, and IL-13Rα2. Recently our group has characterized distinct patterns of STAT6 phosphorylation in two different strains of mice that are associated with disparate outcomes in GCM(12).

The clear role for IL-13 in the development of GCM has been defined in several key studies(3,6,7). Subsequent studies inhibiting or knocking out the IL-4Rα chain or STAT6 support the notion that IL-13 is mediating allergic responses primarily through the type II IL-4 receptor/STAT6 in a classical manner(13-18). Recently we have described an IL-13 dependent upregultion of a GABAergic signaling system in airway epithelium that is required for GCM(19). It is unknown however, if this mechanism of GCM is mediated through the Type II IL-4 receptor(20). We set out to test the hypothesis that allergen mediated upregulation of a GABAergic signaling system in airway epithelium is mediated through the IL-4Rα chain by using a monoclonal antibody to IL-4Rα (anti-IL-4Rα mAb) in a model of brief HDM exposure and examining airway physiology, inflammation, STAT6 phosphorylation, expression of genes containing

STAT6 binding motifs (IL-4, IL-13Rα2), glutamic acid decarboxylase 65/67 (GAD65/67) protein expression, and GCM.

Methods

Animals: Female BALB/c wild type mice, aged 8-10 weeks, were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed in environmentally controlled, specific pathogen-free conditions for a one week acclimatization period and throughout the duration of the studies. All procedures were approved by the Animal Research Ethics Board at McMaster University, and conformed to the NIH guidelines for experimental use of animals.

Brief House Dust Mite Allergen Exposure Model and Intervention Design: Four groups of animals (n=10) were studied as follows **1)** Saline exposed/vehicle intervention, **2)** HDM exposed/vehicle intervention, **3)** HDM exposed/anti-IL-4Rα mAb intervention, and **4)** HDM exposed/control antibody intervention. House dust mite extract (Greer Laboratories, Lenoir, North Carolina) was prepared as described previously(21). Mice received 15μg HDM extract in 25μl PBS volume on Days 1-5 and 8-12 (Figure 1) under gaseous anesthesia via intranasal (IN) route. Control animals received IN saline exposure. Groups 1 and 2 received 250μl of PBS via intraperitoneal (IP) injection as vehicle treatment. Group 3 received 250μl of a 4mg/ml anti-IL-4Rα mAb (Amgen, Seattle, Washington, U.S.A.) via IP injection(22). Group 4 received 250μl of a 4mg/ml control mAb (Amgen, Seattle, Washington, U.S.A.) via IP injection. IP injections were performed on Days 1, 5, and 10 based on pharmacokinetic data (data not shown). Outcome measurements were made 72h post final HDM exposure (Day 15).

Airway Responsiveness: Airway responsiveness was measured using the flexiVent small animal ventilator system (SciReq, Montreal, Quebec, Canada). The single compartment model was employed and total respiratory system resistance (R_{RS}) in response to nebulized saline and increasing doses of methacholine (MCh) (n=10 per group) was assessed.

BAL and Serum analysis: BAL cell differential analysis was performed as previously described(12).

Lung histology: Lungs were dissected and left lobes inflated, fixed in formalin, and embedded in paraffin as previously described (12). Three micron thick sections were stained with Mayer's Hematoxylin/Eosin (H&E) and Periodic Acid-Schiff (PAS) for assessing the presence of tissue eosinophils and goblet cells, respectively. Sections were immunostained using antibodies against phospho-STAT6 (pSTAT6) (Abcam, Cambridge, Mass, U.S.A.) and GAD65/67 (Sigma, Mississauga, Ontario, Canada). All immunostains required antigen retrieval in citrate buffer (pH 6.0) in a steamer at 90°C for 20 min followed by cooling of slides to room temperature for 20 min. Blocking was performed with 1% normal swine serum for 30 min at room temperature and all subsequent reagents were dissolved in this solution. Primary antibodies were diluted at 1:100 and 1:800 for pSTAT6 and GAD 65/67, respectively, and incubated overnight at 4°C. For pSTAT6 staining, biotinylated secondary antibodies were added at 1:100 concentrations, while streptavidin peroxidase was added at 1:600 concentration. Colour

development was performed by placing slides into a 0.4% aminoethylcarbazole/dimethylformamide solution for 30 minutes, initiated by the addition of 4 drops of H_2O_2. Counterstaining was performed with Mayer's Hematoxylin. For GAD65/67 staining, a FITC conjugated antibody to pan-cytokeratin (1:100 dilution, Sigma) was used to visualize epithelium. A Cy3 conjugated secondary antibody was used to detect GAD65/67 (1:100 dilution, Sigma).

Lung morphometry: PAS stained tissue sections were viewed and images collected under 20X objective magnification light microscopy as previously described(12). H&E stained tissue sections were viewed and images collected under 40X objective. Eosinophils were expressed as number/area in a 50μm band extending from the basement membrane of the large primary bronchus as previously described (12). Confocal images of GAD65/67 staining of airway epithelium were analyzed with morphometry as follows: The area of GAD65/67 stain (red) was determined and expressed as a ratio of airway epithelium area. Multiple images were collected (n=6-7) for each animal (n=3) from each group.

Gene Expression Analysis: Following euthanasia, lungs were removed, rinsed in PBS, and immediately immersed in RNAlater (Ambion, Streetsville, ON, Canada) and stored at 4°C overnight as per the manufacturer's recommendations. RNA isolation was performed using a Qiagen MiniPrepKit (Valencia, CA, USA) and concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific,

Wilmington, DE, USA). RNA was transcribed using a standard protocol with an ABI High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was conducted using custom Taqman Low Density Arrays (Applied Biosystems) read on an ABI Prism 7900HT Sequence Detector (Applied Biosystems). Relative quantification was determined using the comparative Ct method. Expression levels were calculated relative to the expression of the control gene hypoxanthine guanine phosphoribosyltransferase (HPRT).

Statistical Analysis: Summary data used in all comparisons are expressed as mean and standard error of the mean (SEM). Comparisons between saline control and OVA exposed mice for each were made using a one way ANOVA with a Newman-Keuls post hoc test. Differences were assumed to be statistically different when the observed p values were less than 0.05.

Results

Anti-IL-4Rα mAb treatment inhibits brief HDM induced AHR and lung eosinophilia

Brief HDM exposure resulted in AHR ($p<0.05$; Figure 2a) illustrated by increased maximum respiratory resistance ($p<0.05$; Figure 2b) measured 72h post final allergen exposure. Intervention with anti-IL-4Rα mAb significantly attenuated HDM induced AHR ($p<0.05$; Figure 2a-b). Control mAb intervention had no effect on AHR.

Brief HDM exposure resulted in increased airway ($p<0.05$; Figure 3a) and tissue ($p<0.05$: Figure 3b) eosinophils. Intervention with anti-IL-4Rα mAb attenuated HDM induced airway and tissue eosinophilia ($p<0.05$; Figure 3), while control mAb had no effect.

Anti-IL-4Rα mAb treatment inhibits brief HDM induced lung STAT6 phosphorylation

We assessed IL-4Rα signalling by examining phosphorylated STAT6 (pSTAT6) levels using immunohistochemistry as previously described for ovalbumin exposure models(12). Representative images of pSTAT6 staining ($n=4$ per group) are illustrated in Figure 4a-d. Brief HDM exposure resulted in pSTAT6 positively staining in small nuclear dense inflammatory cells within the airway parenchyma and beneath the basement membrane (Figure 4a-b). Anti-IL-4Rα mAb intervention inhibited brief HDM induced elevations in pSTAT6 positively stained cells, while control mAb had no effect (Figure 4c-d).

Anti-IL-4Rα mAb treatment inhibits HDM induced upregulation of genes containing STAT6 binding motifs – IL-4 and IL-13Rα2

STAT6 binding motifs are contained in the promoters of various genes including IL-4 and IL-13Rα2(8). Brief HDM resulted in elevated transcript levels encoding IL-4 and IL-13Rα2 that were attenuated with anti-IL-4Rα mAb intervention ($p<0.05$; Figure 5A-B). Control antibody had no effect on HDM induced IL-13Rα2 expression (Figure 5B), but demonstrated a trend ($p=0.09$) for reduction in IL-4 gene transcript levels (Figure 5A).

Brief HDM induces epithelial expression of GAD 65/67 which is inhibited by anti-IL-4Rα mAb treatment

We have previously described upregulation of GAD 65/67 expression in airway epithelium that is involved in an IL-13 dependent GCM in an ovalbumin model of allergen exposure(19). Confocal microscopy of GAD 65/67 expression in airway epithelium following brief HDM exposure demonstrates upregulation following brief HDM allergen exposure (Figure 6A-B). Brief HDM induced upregulation of GAD65/67 is inhibited by anti-IL-4Rα mAb (Figure 6C) but not control antibody (Figure 6D). Morphometric analysis of airway epithelium demonstrated a HDM induced upregulation in area of GAD65/67 staining that was significantly reduced by anti-IL-4Rα mAb treatment (Figure 6E).

Anti-IL-4Rα mAb treatment inhibits brief HDM induced GCM and mucin gene expression

Brief HDM exposure resulted in elevations in PAS positively stained cells with goblet cell morphology as compared to saline exposure (Figure 7a-b). Anti-IL-4Rα mAb treatment inhibited the development of PAS positively stained goblet cells, although some PAS positive cells without goblet cell morphology were observed (Figure 7c). Control antibody had no effect (Figure 7d).

Quantification of positively stained PAS cells revealed a HDM induced increase that was significantly attenuated by anti-IL-4Rα mAb treatment ($p<0.05$; Figure 7e). Control antibody had no treatment effect.

Consistent with GCM, brief HDM induced increases in MUC5AC ($p<0.05$; Figure 8A) and Gob5 gene expression ($p<0.05$; Figure 8B). Anti-IL-4Rα mAb treatment significantly attenuated brief HDM induced changes in mucin gene expression ($p<0.05$; Figure 8A-B).

Discussion

The present results extend our previous findings of an IL-13 dependent GABAergic signaling system in airway epithelium involved in GCM in an ovalbumin model of allergen exposure(19). We demonstrate by using brief HDM exposure that the upregulation of a GABAergic system in airway epithelium is conserved in different models of allergen exposure. Furthermore, we demonstrate that brief HDM results in phosphorylation of STAT6, a key signaling molecule downstream of IL-4Rα. Finally, we provide evidence using an anti-IL-4Rα mAb intervention that HDM induced responses signal through IL-4Rα to produce elevations in STAT6 phosphorylation, GAD65/67 expression, GCM, and mucus gene expression.

The signaling cascade of IL-13 leading to upregulation of the GABAergic system in airway epithelium and GCM is not fully elucidated (20). IL-13 is capable of signaling through a heterodimer of IL-4Rα and IL-13Rα1 (23) or through IL-13Rα2 (24). Previous reports using IL4Rα -/- mice demonstrate that this receptor is necessary for GCM following ovalbumin exposure(6,18). Our data using a pharmacological inhibitor of the IL-4Rα is consistent with these findings and extends them by demonstrating that GAD 65/67 expression in models of allergen exposure is regulated through the IL-4Rα and downstream STAT6 pathway.

STAT6 is transcription factor downstream of receptor chain IL-4Rα and can be phosphorylated as a result of IL-4 or IL-13 signaling(8). IL-4 signals through STAT6 using the IL-4Rα and the common γ receptor chain, while IL-13 uses the IL-13Rα1 chain in place of common γ chain. Using ovalbumin exposure models we have previously

demonstrated that observed STAT6 phosphorylation is dependent on IL-13(12). Our findings of elevated pSTAT6 levels following brief HDM exposure are consistent with those observed in ovalbumin models. It remains to be determined if changes in pSTAT6 levels in HDM models are IL-13 dependent, although the staining pattern, cell morphology, and expression levels are similar to IL-13 dependent STAT6 elevations observed in OVA models (12).

The functional consequences of IL-4Rα blockade have been reported using models of ovalbumin exposure with knockout of IL-4Rα (6,18), while indirectly explored in STAT6 deficient animals (15,25). Classical knockout animal studies suffer from the potential for compensatory mechanisms and potentially confounding animal development issues(26) although the use of cre-lox inducible knockouts circumvent these problems(27). Using our brief model of HDM exposure we support the role of IL-4Rα in the development of allergen induced AHR, although the precise mechanisms by which this occurs are unknown. We provide a proof of concept study for IL-4Rα intervention in chronic models of HDM exposure which will allow a clinically relevant assessment of this pathway, including treatment initiation following establishment of airway remodeling and sustained AHR(28). Chronic models of IL-4Rα intervention using anti-sense technology have observed attenuation in the allergic phenotype and AHR – but a residual GCM still exists(17).

Similar to Karras et al. (17), we did not see complete attenuation of GCM in our anti-IL-4Rα mAb treated mice. It is possible that our pharmacological inhibitor did not achieve complete receptor blockade of brief HDM induced signaling through the IL-4Rα

pathway, although published reports using this reagent at lower doses had efficacy in an allergic gastrointestinal model(22). We examined this possibility by assessing IL-4 and IL-13Rα2 gene transcript levels, two genes reported to be regulated by the IL-4Rα/STAT6 pathway(8). Complete inhibition of brief HDM induced elevations in IL-4 and IL-13Rα2 gene transcript suggest that we had sufficient levels of inhibitor to block gene expression.

We have previously reported on a GABAergic signaling system in airway epithelium involved in GCM that is dependent on IL-13(19). Ovalbumin exposure models were used in animal studies to support these findings. As ovalbumin exposure models require artificial sensitization(29), may lead to allergen tolerance(30), and are not reflective of clinically relevant allergens(31-33), we sought to examine whether upregulation of the GABAergic signaling system was conserved for more relevant allergens. Using a model of brief HDM exposure that requires no artificial sensitization, results in no allergen tolerance (21,32,34), and is of clinical relevance(35), we demonstrate that GABAergic upregulation in airway epithelium is conserved among two common models of allergen exposure.

Our findings provide evidence to support the role of an IL-4Rα dependent GABAergic signaling system in airway epithelium involved in GCM in response to allergen exposure. Using an anti-IL-4Rα mAb intervention we demonstrate for the first time that allergic responses signal through this receptor chain to upregulate GAD 65/67 expression in airway epithelium and induce GCM. Consistent with inhibition of IL-4Rα signaling, we observe reduced phosphorylated STAT6 protein levels with anti-IL-4Rα

mAb treatment during HDM exposure. These findings support the pursuit of IL-4Rα inhibitors for the management of allergen induced GCM and associated airway dysfunction.

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Figures

Figure 1: Allergen sensitization and intervention dosing protocol. Four groups of animals (n=10) were studied (see methods). Saline exposed/vehicle intervention, HDM exposed/vehicle intervention, HDM exposed/anti-IL-4Rα mAb intervention, and HDM exposed/control antibody intervention. Black circles: Intranasal (IN) HDM and intraperitoneal injection of vehicle, anti-IL4Ra-mAb intervention, or control antibody intervention. Open circles: IN HDM exposure. Grey circles: Rest day.

Figure 2: Airway responsiveness to nebulized methacholine. **A)** Dose response curve. Black circles/solid line: Saline exposed/vehicle intervention (see methods). Black squares/solid line: HDM exposed/vehicle intervention. Open circles/dashed line: HDM exposed/anti-IL-4Rα mAb intervention. Open squares/dashed line:HDM exposed/control antibody intervention. **B)** Maximum airway resistance. Open white bar: Saline exposed/vehicle intervention. Solid black bar: HDM exposed/vehicle intervention. Horizontal dashed white bar: HDM exposed/anti-IL-4Rα mAb intervention. Diagonal dashed white bar: HDM exposed/control antibody intervention. * = significantly different from saline exposed/vehicle intervention ($p<0.05$). & = significantly different from HDM exposed/vehicle intervention ($p<0.05$).

Figure 3: Airway eosinophilia. **A)** BAL eosinphils. **B)** Tissue eosinophils. Open white bar: Saline exposed/vehicle intervention. Solid black bar: HDM exposed/vehicle

intervention. Horizontal dashed white bar: HDM exposed/anti-IL-4Rα mAb intervention. Diagonal dashed white bar: HDM exposed/control antibody intervention. * = significantly different from saline exposed/vehicle intervention ($p<0.05$). & = significantly different from HDM exposed/vehicle intervention ($p<0.05$).

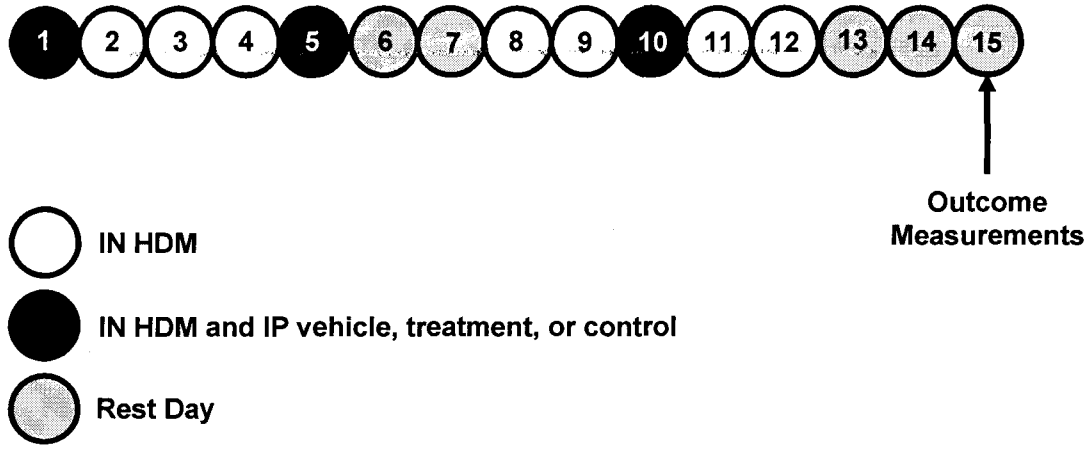
Figure 4: Expression of phosphorylated STAT6 (pSTAT6) protein following brief HDM exposure. Panels **A-D**: representative images of pSTAT6 immunohistochemistry of mouse lung. Immunostain was developed with aminoethylcarbazole producing a red/brown stain on pSTAT6 positive cells. Slides were counterstained with Mayer's Hematoxylin. **A)** Saline exposed/vehicle intervention. **B)** HDM exposed/vehicle intervention. **C)** HDM exposed/anti-IL-4Rα mAb intervention. **D)** HDM exposed/control antibody intervention. Red arrows point to positively stained cells.

Figure 5: Relative gene expression of genes containing STAT6 binding motifs following brief HDM exposure. Gene expression is expressed relative to HPRT (see methods). **A)** IL-4 **B)** IL-13Rα2. Open white bar: Saline exposed/vehicle intervention. Solid black bar: HDM exposed/vehicle intervention. Horizontal dashed white bar: HDM exposed/anti-IL-4Rα mAb intervention. Diagonal dashed white bar: HDM exposed/control antibody intervention. * = significantly different from saline exposed/vehicle intervention ($p<0.05$). & = significantly different from HDM exposed/vehicle intervention ($p<0.05$).

Figure 6: Expression of GAD65/67 protein in mouse lung using confocal microscopy following brief HDM exposure. Epithelial cells were identified by immunostaining with pan-cytokeratin (green). GAD 65/67 immunostaining is red. Representative images (n=5 per group). **A)** Saline exposed/vehicle intervention. **B)** HDM exposed/vehicle intervention. **C)** HDM exposed/anti-IL-4Rα mAb intervention. **D)** HDM exposed/control antibody intervention. **E)** Morphometric analysis of GAD65/67 staining. Multiple images of airways (n=6) from three animals from each group were analyzed. The area of GAD65/67 staining was normalized to epithelium area. * = significantly different from saline exposed/vehicle intervention ($p<0.05$). & = significantly different from HDM exposed/vehicle intervention ($p<0.05$).

Figure 7: Goblet cell analysis following brief HDM exposure. Representative photomicrographs of PAS positively stained slides for **A)** saline exposed/vehicle intervention, **B)** HDM exposed/vehicle intervention, **C)** HDM exposed/anti-IL-4Rα mAb intervention, and **D)** HDM exposed/control antibody intervention. **E)** Quantification of PAS positively stained cells. Open white bar: Saline exposed/vehicle intervention. Solid black bar: HDM exposed/vehicle intervention. Horizontal dashed white bar: HDM exposed/anti-IL-4Rα mAb intervention. Diagonal dashed white bar: HDM exposed/control antibody intervention. * = significantly different from saline exposed/vehicle intervention ($p<0.05$). & = significantly different from HDM exposed/vehicle intervention ($p<0.05$).

Figure 8: Relative gene expression of mucus related genes following brief HDM exposure. Gene expression is expressed relative to HPRT (see methods). **A)** MUC5AC **B)** Gob5. Open white bar: Saline exposed/vehicle intervention. Solid black bar: HDM exposed/vehicle intervention. Horizontal dashed white bar: HDM exposed/anti-IL-4Rα mAb intervention. Diagonal dashed white bar: HDM exposed/control antibody intervention. * = significantly different from saline exposed/vehicle intervention ($p<0.05$). & = significantly different from HDM exposed/vehicle intervention ($p<0.05$).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Outcome Measurements
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Rest Day

Figure 1

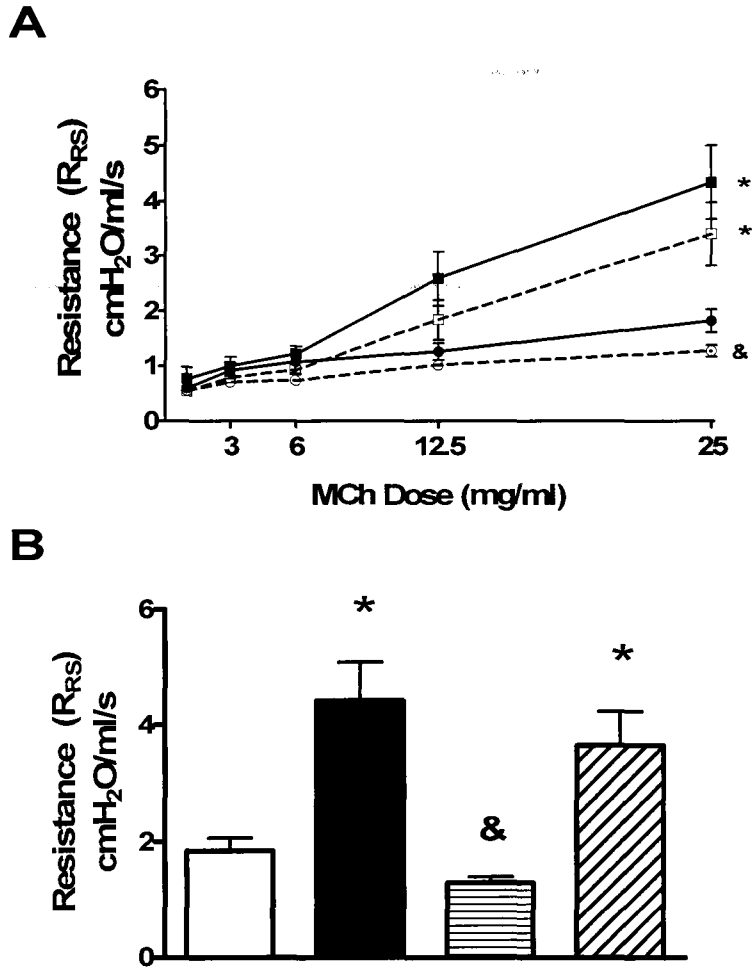
A
Resistance (R_{RS}) $cmH_2O/ml/s$
0
1
2
3
4
5
6
3
6
12.5
25
MCh Dose (mg/ml)
*
*
&
B
Resistance (R_{RS}) $cmH_2O/ml/s$
0
2
4
6
*
&
*

Figure 2

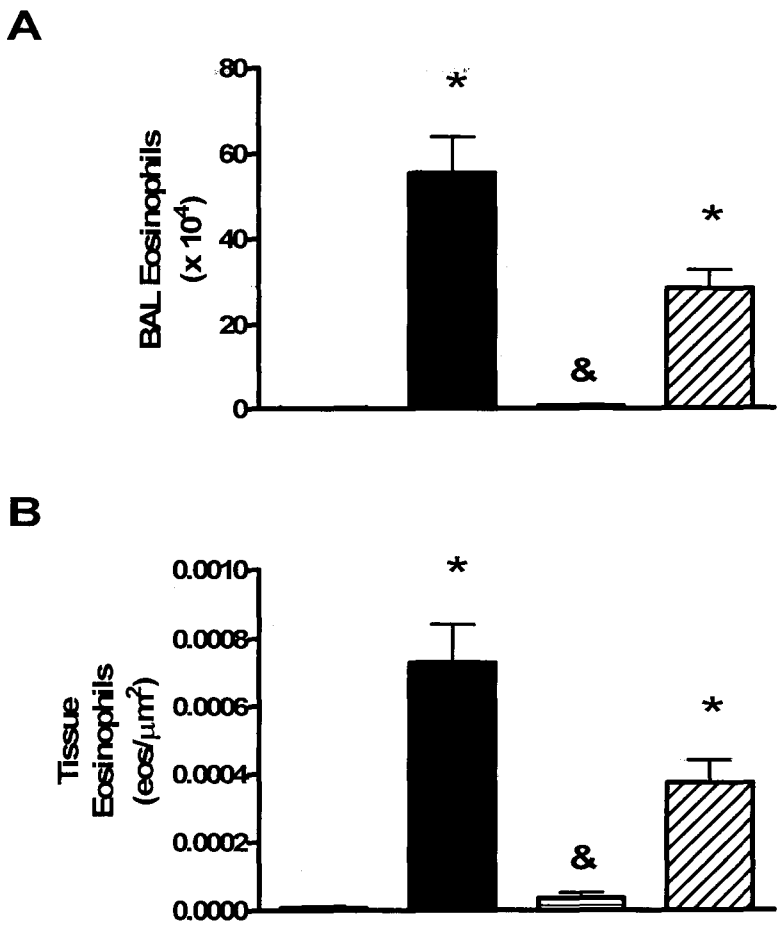
A
BAL Eosinophils (x 10⁴)
80
60
40
20
0
*
&
*
B
Tissue Eosinophils (eos/μm²)
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
*
&
*

Figure 3

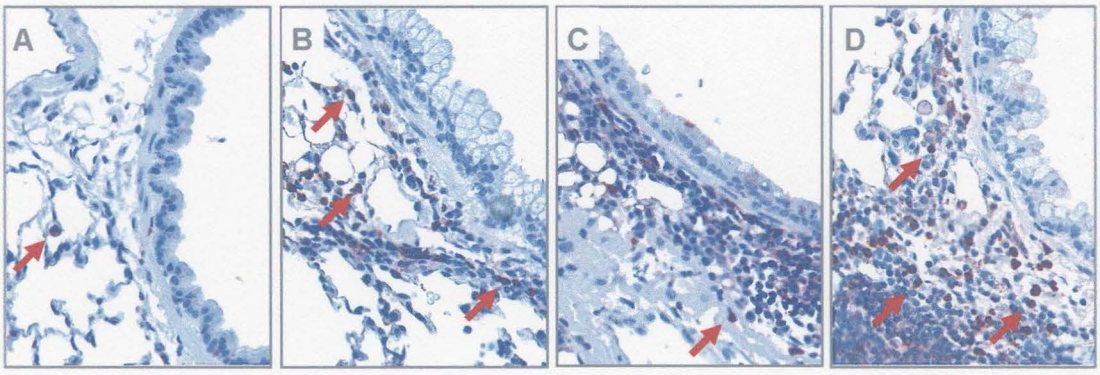

Figure 4

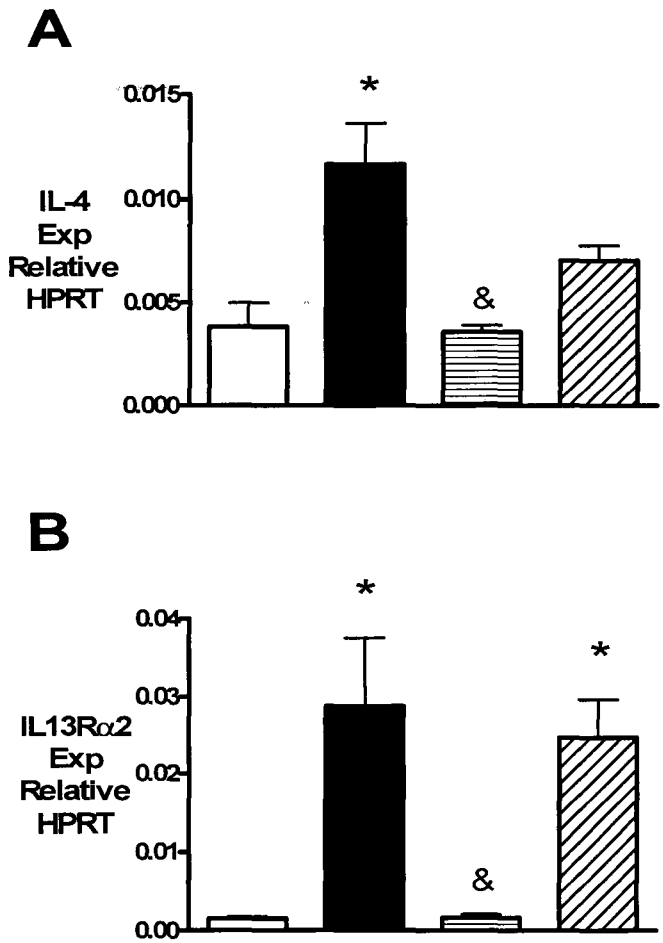
A
IL-4 Exp Relative HPRT
0.015
0.010
0.005
0.000
*
&
B
IL13Rα2 Exp Relative HPRT
0.04
0.03
0.02
0.01
0.00
*
&
*

Figure 5

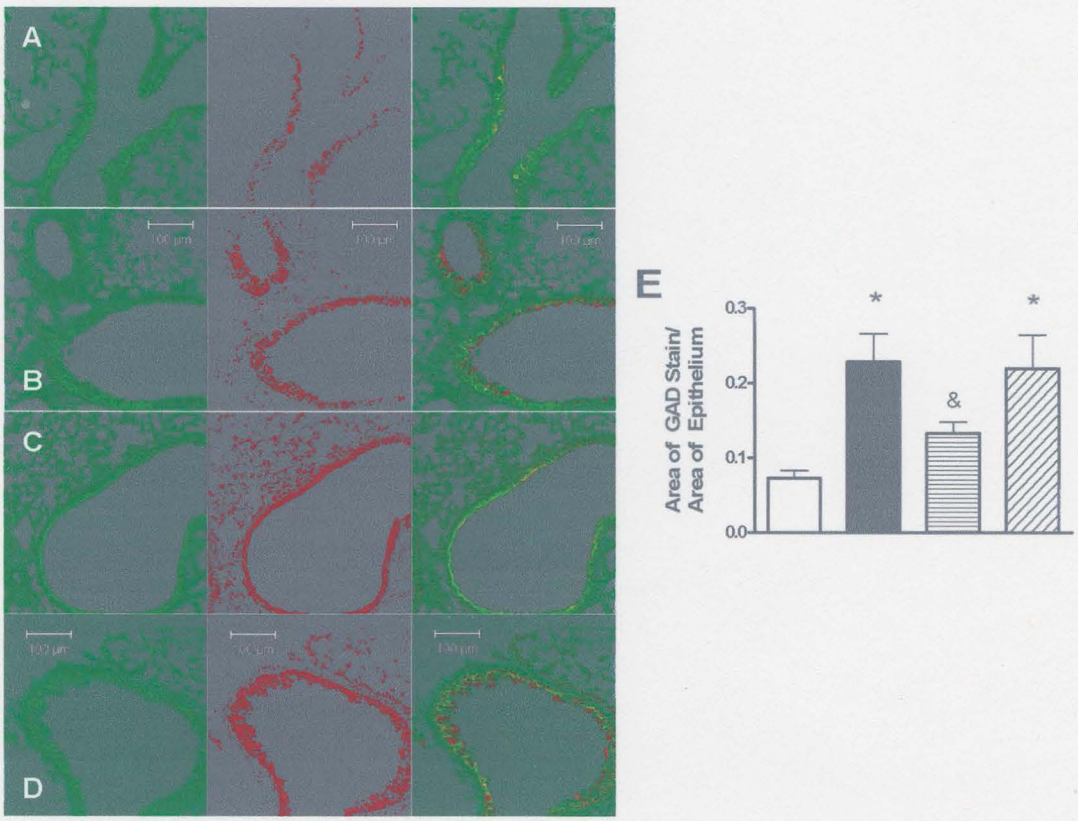

Figure 6

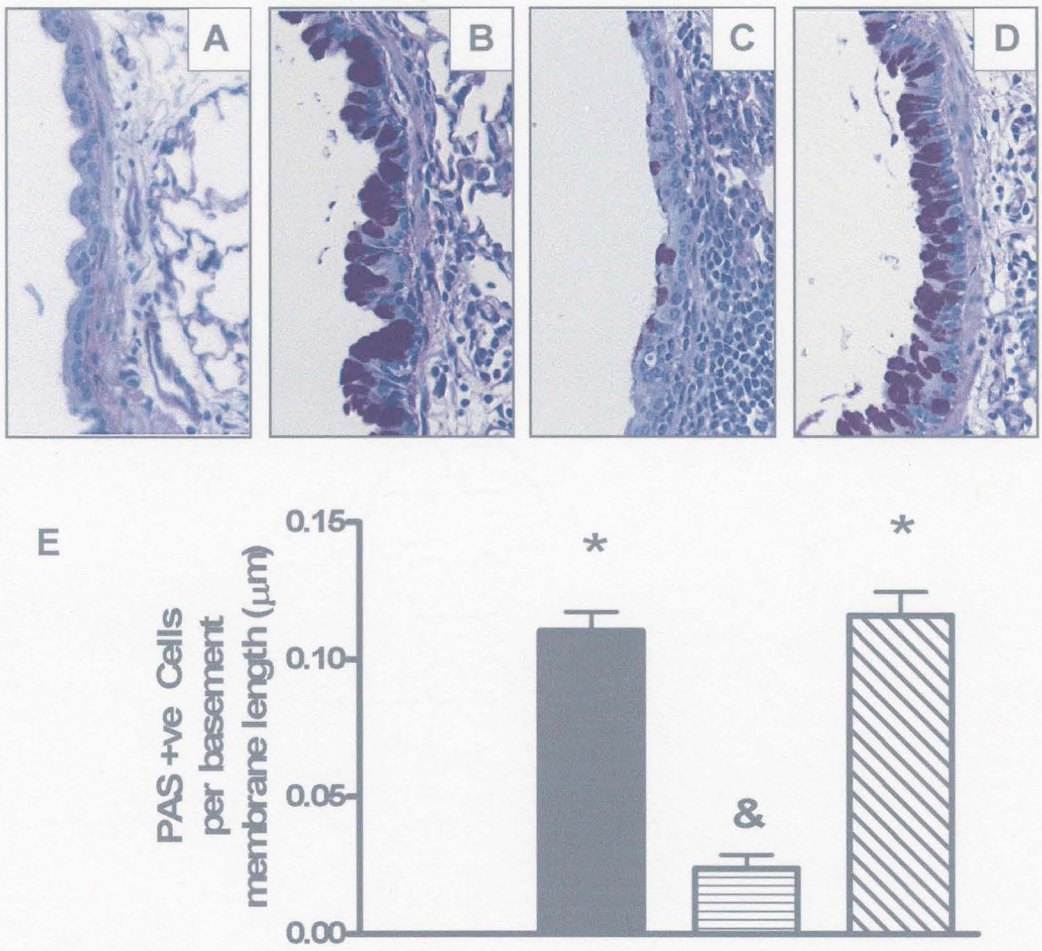
A
B
C
D
E
PAS +ve Cells per basement membrane length (μm)
0.15
0.10
0.05
0.00
*
&
*

Figure 7

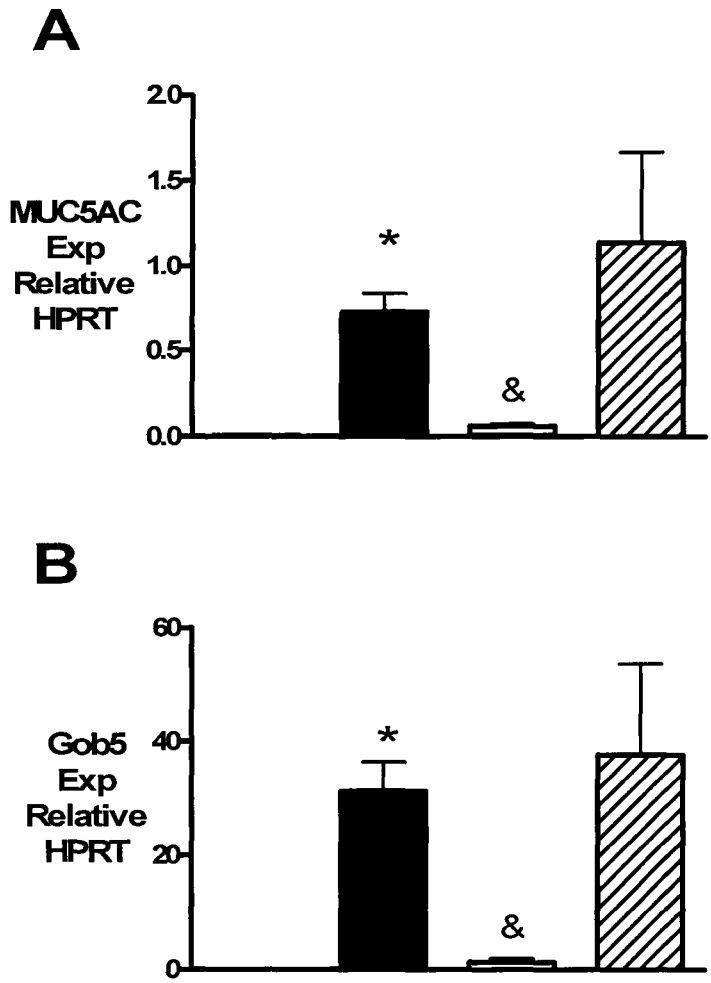
A
2.0
1.5
1.0
0.5
0.0
MUC5AC
Exp
Relative
HPRT
*
&
B
60
40
20
0
Gob5
Exp
Relative
HPRT
*
&

Figure 8

CHAPTER 6

THE ROLE OF PLATELET DERIVED GROWTH FACTOR-BB IN AIRWAY SMOOTH MUSCLE PROLFIRATION IN MOUSE LUNG

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Jeremy Hirota's contributions:

As primary author I, J. Hirota, was responsible for conceiving, developing, and managing the entire study. This included tissue collection, processing, immunohistochemistry, morphometrics, immunofluorescence, and gene expression. K. Ask procured adenovirus reagents and aided in gene expression analysis. L. Farkas was integral in providing methods and expertise in immunofluorescence. J.C. Rodriguez-Lecompte created the PDGF-BB adenovirus construct used in the experiments. M. Kolb reviewed the manuscript and provided reagents and lab infrastructure for gene expression analysis. Analysis, statistics, figure generation, manuscript preparation, and submission were performed by me with guidance from M.D. Inman. M.D. Inman is the senior author and primary investigator of the lab that the work was completed in.

The role of platelet derived growth factor-BB in airway smooth muscle proliferation in mouse lung

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Key Words :
Asthma, Airway Remodeling, Gene expression, Budesonide

Abstract

Background : ASM hyperplasia in asthma contributes largely to functional changes and investigating the mechanisms behind proliferation of these cells may provide therapeutic benefit. PDGF-BB is a well known airway smooth muscle (ASM) mitogen in vitro, but has yet to be explored using in vivo mouse models of chronic allergen exposure where ASM hyperplasia is observed.

Objectives : Our objectives were two fold. i) To determine the role of PDGF-BB in regulating genes transcripts encoding contractile proteins, ASM proliferation, and airway physiology, using an adenovirus overexpression system. ii) To evaluate PDGF-BB in a model of chronic allergen exposure in relation to expression of gene transcripts encoding contractile proteins and ASM hyperplasia, and assess the consequences of budesonide intervention on these outcomes.

Methods : We used adenovirus technology to selectively overexpress PDGF-BB in mice. Outcome measurements were performed 7d post exposure and included airway physiology, real time – RT-PCR measurements of gene transcript levels, proliferating cell nuclear antigen (PCNA) staining, and ASM quantification. Additionally we investigated the role of PDGF-BB in a model of chronic allergen exposure to ovalbumin (OVA) with a budesonide intervention arm. Outcome measurements were performed 24h and 4wk post chronic allergen exposure.

Results : PDGF-BB overexpression resulted in airway hyperresponsiveness, proliferation in ASM cells, positive PCNA stained ASM cells, and a reduction in genes encoding contractile proteins. Chronic allergen exposure resulted in increased BAL PDGF-BB

levels, ASM hyperplasia, and a selective reduction in gene transcripts encoding contractile proteins. Budesonide treatment normalizes allergen induced changes in PDGF-BB levels and gene transcript levels, but does not inhibit ASM hyperplasia.

Conclusion : The studies presented demonstrate several important findings in relation to ASM hyperplasia. We demonstrate for the first time in vivo, a PDGF-BB dependent reduction of genes encoding contractile proteins with concomitant ASM hyperplasia. Furthermore, allergen exposure results in changes in lung PDGF-BB biology that are associated with reductions in gene transcript levels encoding contractile proteins and ASM hyperplasia. While budesonide significantly attenuated changes in lung PDGF-BB biology and gene transcript levels, ASM hyperplasia was resistant to steroid intervention.

Introduction

Asthma is a disease defined by variable airflow obstruction and characterized by airway hyperresponsiveness (AHR), inflammation, and remodeling. The relationship between these three processes is not completely understood, although it is generally accepted that both inflammation and remodeling contribute to AHR. The association between airway inflammation and airway remodeling is strengthened by *in vitro* and *in vivo* studies that have demonstrated the ability of several inflammatory mediators and growth factors to be potent inducers of indices of remodeling including goblet cell metaplasia (1), collagen production (2), and airway smooth muscle (ASM) proliferation (3). Although the relative contribution of each remodeling index to functional changes has not been clearly delineated using *in vivo* models or clinical studies, mathematical modeling has emphasized the central role that increased ASM mass plays in airway narrowing(4). Additionally, increased ASM mass in clinical biopsies of asthmatics(5,6) and the increased proliferative capacity of asthmatic ASM cells in culture have been documented(7), Finally, recent efficacious non-pharmacological intervention aimed at selectively ablating ASM (8) add to the evidence supporting the central role of ASM in asthma related functional changes.

The molecular mechanisms by which ASM proliferates may be governed by several growth factors including insulin like growth factor(9,10), basic fibroblast growth factor(11), transforming growth factor β1(12), and platelet derived growth factors (3). Of these growth factors, platelet-derived growth factor (PDGF) BB has been studied extensively in culture and is used frequently as a positive control growth stimulus for

ASM proliferation(13,14). In vascular smooth muscle cells it has been demonstrated that PDGF-BB signaling leads to changes in transcription factors that bind to promoter sequences upstream from smooth muscle specific genes, including α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SM-MHC), calponin, and desmin(15). In vascular smooth muscle cells, PDGF-BB signaling results in downregulation of the aforementioned gene transcript levels and changes in cell phenotype associated with proliferation(15,16). It is currently unknown if PDGF-BB induced changes in cell phenotype due to alterations in gene transcript expression occur in ASM cells, or if this event occurs in the context of allergic inflammation.

Clinical studies have been performed investigating PDGF-BB levels in the context of asthma, but are limited to observational studies and have produced conflicting results(17-19). In addition to the absence of experimental studies in humans, the direct role of PDGF-BB in ASM proliferation has not been explored using any animal models such as direct over-expression with adenovirus or chronic allergen exposure.

In this manuscript we tested the hypothesis that PDGF-BB is sufficient for induction of ASM hyperplasia *in vivo*. We explored this hypothesis by quantifying gene expression levels for contractile proteins, ASM cell proliferation, and quantification of ASM cells, in two distinct mouse models. Our first proof of concept model involved selective over-expression of PDGF-BB in mouse airway epithelium using adenovirus gene transfer to validate *in vitro* studies supporting the pro-mitogenic role of PDGF-BB on ASM(20). Subsequently, we determined whether changes in PDGF-BB lung expression occurred in chronic allergen exposure models and whether these were

associated with changes in gene expression levels for contractile proteins and ASM hyperplasia. As selective anti-PDGF-BB signaling inhibitors are unavailable (21), we also included a non specific budesonide intervention arm to investigate if any changes in PDGF-BB lung expression are steroid sensitive, and whether this would in turn influence ASM phenotype.

Methods

Animals: Female BALB/c, aged 8-10wks, were purchased from Harlan (Indianapolis, IN). All mice were housed in environmentally controlled, specific pathogen-free conditions for a one week acclimatization period and throughout the duration of the studies. All procedures were approved by the Animal Research Ethics Board at McMaster University, and conformed to the CIHR guidelines for experimental use of animals.

Adenovirus Mediated Overexpression of PDGF-BB: Full-length of rat PDGF-BB cDNA was cloned in a human replication-deficient type-5 adenovirus to make the expressed protein product constitutively and biologically active(2). The E1 region was replaced by the human cytomegalovirus promoter, driving expression of PDGF-BB followed by the SV40 polyadenylation signal. The resulting replication-deficient virus (AdPDGF-BB) was amplified and purified by cesium chloride (CsCl) gradient centrifugation and concentrated using a Sephadex PD-10 chromatography column, and finally plaque-titered on 293 cells(22). The control vectors, AdDL, with no insert in the deleted E1 region was produced by similar methods. Mice received a single intratracheal (IT) instillation of $5x10^8$ plaque forming units (pfu) of either PDGF-BB adenovirus (AdV) (treatment) or null AdV (control) on Day 0. Outcome measurements were performed on Day 7 post AdV IT instillation (Figure 1A).

Allergen Sensitization, Exposure, and Budesonide Treatment: Mice were sensitized as described previously(23). Briefly, all mice received intraperitoneal (IP) injections of ovalbumin (OVA) precipitated with aluminium potassium sulfate on Days 1 and 11 and IN OVA on Day 11. Following sensitization, mice were subjected to an allergen exposure protocol with multiple time points for outcome measurements (Figure 1B). Chronic allergen exposure was comprised of six 2-day periods of intranasal ovalbumin (IN OVA) (100 μg in 25 μl saline) administration each separated by 12 days. Exposures started on Days 19 and 20. Outcome measurements were made at the following time points; (i) 24hrs following the complete chronic allergen exposure protocol (Day 91), and (ii) 4wks following the complete chronic allergen exposure (Day 118). A separate budesonide intervention study arm was designed with outcomes performed at Day 91 and Day 118. Budesonide suspension (in 1% caroboxymethylcellulose vehicle) was administered once daily (S.I.D.) via IP injection (3mg/kg) throughout the period of allergen exposure (Days 19-91) and during both allergen exposure and allergen avoidance (Days 19-118) (Figure 1B). Control mice were treated in identical fashion but received vehicle alone.

Bronchoalveolar Lavage (BAL) and Analysis: BAL and ELISAs were performed for PDGF-BB in BAL supernatant (BD Biosciences; San Diego, CA and R&D Systems; Minneapolis, MN) as previously described(23).

Lung histology, immunohistochemistry, and immunofluorescence: Lungs were dissected, removed, and left lobes inflated with 10% formalin with a pressure of 25cm H_2O, ligated at the trachea, and fixed in 10% formalin for 24hrs. Sections were immunostained using antibodies against PCNA (Cell Signaling, Danvers, MA, USA) α-SMA, and smooth muscle myosin heavy chain (SM-MHC) (Dako, Mississauga, Canada). For PCNA immunostaining, tissues were adhered to poly-L-lysine coated slides, placed in a 70°C oven for 35min, deparaffinized, endogenous peroxidase blocked, and antigen retrieval performed using steaming in citrate buffer. Blocking was performed with 1% normal swine serum before application of optimized primary antibody dilution (1:100 for both antibodies). Biotinylated secondary antibodies, horseradish peroxidase streptavidin (HRP-SA), aminoethylcarbazole, and counterstain with hematoxylin were used to develop staining colour. Dual staining of sections with PCNA and α-SMA was performed with FITC and TRITC labelled secondary antibodies, respectively. Dual staining of sections with SM-MHC and α-SMA was performed with FITC and TRITC labelled secondary antibodies, respectively. All immunofluorescence protocols used a DAPI nuclear stain (Dako, Mississauga, Canada).

Lung morphometry: Quantifying the ASM cell number was performed with a digital image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, Ontario, Canada). SM-MHC and α-SMA dual stained tissues were analyzed as follows. 1) Individual images of SM-MHC (FITC), α-SMA (TRITC), and nuclei (DAPI) were taken from the large primary bronchus in the left lobe. 2) Merged images of FITC,

TRITC, and DAPI channels were created, saved, and analyzed for area of colococalized FITC/TRITC staining. 3) The number of nuclei in the FITC/TRITC colocalized stain was recorded as an index of cell number. 4) The basement membrane length was determined in microns. 5) FITC/TRITC colocalized area was normalized to basement membrane length to yield an index of airway smooth muscle area. 6) The number of DAPI nuclei colocalized to the FITC/TRITC dual stain was normalized to basement membrane length to yield an index of airway smooth muscle cell number. 7) The number of DAPI nuclei colocalized to FITC/TRITC dual stain area was normalized to area of FITC/TRITC dual stain area to yield and index of cell size.

Gene Transcript Level Analysis: Gene transcript level analysis was performed 7d post AdV exposure, and 24hrs or 4wk post chronic allergen exposure on isolated airway segments with parenchyma and vasculature removed (Figure 2). Our preparation removes all visible vasculature prior to analysis of gene transcripts encoding contractile proteins (Figure 2). Following euthanasia, lungs were removed, rinsed in PBS, and immediately immersed in RNAlater (Ambion, Streetsville, ON, Canada) and stored at 4°C overnight as per the manufacturer's recommendations followed by macrodissection and airway isolation the next day. RNA isolation was performed using a Qiagen MiniPrepKit (Mississauga, ON, Canada) and concentration and purity were assessed by optical density measurements on a spectrophotometer (Nanodrop Products, Wilmington, DE, U.S.A.). RNA was transcribed using a standard protocol (Invitrogen Life Technologies, Burlington, ON, Canada). Quantitative real-time PCR was conducted

using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). Negative control samples (no template or no reverse transcriptase) were run concurrently. Relative quantification was performed (ddCT method) with results normalized to GAPDH and all values expressed as a fold increase over a naïve lung. GAPDH expression was not significantly different between any treatment groups (data not shown). Assay on Demand products for α-SMA (Mm01546133), smooth muscle myosin heavy chain II (SM-MHC II) (Mm004430133), calponin (Mm00487032), desmin (Mm00802455), and GAPDH (mM99999915) were purchased from Applied Biosystems.

Airway Responsiveness: Airway responsiveness was measured using the flexiVent small animal ventilator system (SciReq, Montreal, Quebec, Canada). The single compartment model was employed and total respiratory system resistance (R_{RS}) in response to nebulized saline and increasing doses of methacholine (MCh) (n=10 per group) was assessed.

Statistical Analysis: Summary data used in all comparisons are expressed as mean and standard error of the mean (SEM). Comparisons between groups were performed with a one-way ANOVA using a Bonferroni correction post hoc test. Differences were assumed to be statistically different when the observed p values were less than 0.05.

Results

PDGF-BB is sufficient for changes in expression of gene transcript levels for contractile proteins, ASM proliferation, and airway hyperresponsiveness in BALB/c mice

We utilized an adenovirus vector to over express PDGF-BB in murine airways to investigate patterns of ASM specific gene expression and ASM cell proliferation. This method resulted in transient elevation of target protein at 7d post exposure which returned baseline levels at 14d (data not shown).

Relative gene expression analysis 7d post AdPDGF-BB intratracheal instillation resulted in significantly lower levels of α-SMA and SM-MHC II gene transcripts as compared to AdNull treated (n=5) (Figure 3a-b; $p<0.05$). PDGF-BB effects on calponin ($p=0.08$) and desmin ($p=0.13$) were not statistically significant (Figure 3c-d).

Proliferating cell nuclear antigen (PCNA) staining was performed to test the hypothesis that decreased transcript levels for contractile proteins in the presence of PDGF-BB are associated with ASM cell hyperplasia. PCNA staining in AdNull treated animals revealed no positive stained ASM cells in small or large airways (Figure 4a-b). In contrast, PCNA staining in AdPDGF-BB treated animals showed positively stained ASM cells in both large and small airways (Figure 4d-e). Dual staining immunofluorescence with PCNA (FITC) and α-SMA (TRITC), counterstained with DAPI also demonstrate PCNA positive ASM cells in AdPDGF-BB, but not Ad Null, treated animals (Figure 4c,f).

The presence of positively stained PCNA cells may indicate DNA damage/repair mechanisms have been active in addition to cell proliferation (24). For this reason, we quantified ASM cell number following PDGF-BB over-expression in our mice. Quantification of ASM cells 7d following AdPDGF-BB instillation revealed an increase in area of ASM (Figure 5a; $p<0.05$) and ASM cell number (Figure 5b; $p<0.05$), but not in an index of cell size (Figure 5c).

PDGF-BB induced changes in ASM related gene transcript levels, mass, and cell number were associated with increased airway responsiveness to nebulized methacholine (Figure 6; $p<0.05$).

Airway lumen PDGF-BB levels are upregulated immediately following chronic allergen exposure and are steroid sensitive

Chronic OVA exposure resulted in elevated levels of endogenous BAL PDGF-BB protein 24hr post exposure as compared to saline control animals (Figure 7a; $p<0.05$). Daily budesonide treatment resulted in almost complete normalization of the allergen induced response, with a strong trend towards to reduction as compared to OVA (Figure 7a; $p=0.05$).

A 4wk period of recovery following chronic allergen exposure resulted in previously elevated BAL PDGF-BB levels returning to levels observed in saline control animals, and was not affected by budesonide treatment (Figure 7b).

Allergen induced changes in PDGF-BB levels are associated with a decrease in gene transcript levels for contractile proteins and are steroid sensitive

Analysis of relative gene transcript levels 24h and 4wk post chronic allergen exposure was performed (Figure 8). At both 24h and 4wk post chronic allergen exposure time-points, allergen exposure resulted in lower levels of α-SMA, SM-MHC II, calponin, and desmin gene transcript(Figure 8a-d; $p<0.05$). Budesonide treatment resulted in the reduction of allergen-induced changes in gene transcript levels for α-SMA, calponin, and desmin (Figure 8a,c-d; $p<0.05$). While budesonide treatment did not affect SM-MHC II transcript expression 24h post allergen exposure, a significant effect was observed 4wk post exposure (Figure 8b; $p<0.05$).

Allergen induced changes in PDGF-BB biology are associated with ASM hyperplasia that is not steroid sensitive

ASM quantification was performed 24h and 4wk post chronic allergen exposure demonstrating a significant increase in ASM number at both time-points (Figure 9; $p<0.05$). Budesonide treatment did not inhibit the allergen induced increases in ASM number 24hr or 4wk post chronic allergen exposure (Figure 9a-b).

Discussion

In this manuscript, using two distinct mouse models we explored the *in vivo* role of PDGF-BB in regulating gene transcript levels for contractile proteins as a marker of cell phenotype and ASM cell proliferation. PDGF-BB over-expression in mouse lung airway epithelium selectively reduced gene transcript levels for contractile proteins, induced ASM hyperplasia, which was accompanied by increased airway responsiveness. We further demonstrate that changes in lung PDGF-BB levels are observed during chronic allergen exposure, and are associated with reductions in contractile gene transcript levels and ASM hyperplasia. Daily budesonide intervention is capable of significantly inhibiting allergen induced changes in lung PDGF-BB biology and gene transcript levels, but not ASM hyperplasia. Our studies suggest that PDGF-BB is capable of inducing ASM hyperplasia *in vivo* but alternate pathways may be more relevant in allergen induced increases in ASM content. These findings may have important implications in the development of therapeutics targeting ASM hyperplastic mechanisms.

The observation of increased muscle mass in airway walls of asthmatics may be the result of both ASM hyperplasia and hypertrophy(5,6), with the relative contribution of each growth mechanism to pathology unknown. Because of the established role of PDGF-BB in ASM hyperplasia *in vitro*(3,25), we chose to investigate the mechanisms of ASM hyperplasia *in vivo* using two distinct exposure models in mice. Our AdPDGF-BB over-expression model demonstrates increased ASM number, PCNA positive ASM cells, but not changes in size, consistent with a hyperplasia process. We could not locate PCNA positive ASM cells in our chronic allergen exposure model at either 24h or 4wk

post exposure. The life-cycle of PCNA is temporal and changes throughout the cell cycle and is significantly elevated in a narrow time frame during cell division, hence the suitable use as a marker of cell proliferation(26). It is possible that at the time-points chosen in our chronic allergen model, PCNA expression in ASM cells was at levels consistent with a quiescent cell phenotype that had completed cell division at some time prior to analysis. Despite that lack of PCNA stained ASM cells in our chronic allergen exposed animals, we detected significant increases in ASM cell number at both time-points suggesting ASM proliferation had previously occurred.

Precise *in vivo* mechanisms responsible for ASM hyperplasia are not understood, although *in vitro* ASM studies have provided much insight. PDGF-BB is commonly used as a potent ASM mitogen in culture systems although direct *in vivo* evidence to support its role has not been demonstrated(13,20). In vascular smooth muscle PDGF-BB signalling has been demonstrated to activate downstream ERK (11) that leads to increases in Elk-1 activity via phosphorylation and subsequent binding of phospho-Elk-1 to C(AT rich)G (CaRG) sequences(15). It has been suggested that binding of phospho-Elk-1 to the smooth muscle CaRG sequences in promoter regions inhibits SRF-myocardin formation, promoter activation, gene expression, and results in phenotype changes required for proliferation of smooth muscle cells(15,16). Our results demonstrate for the first time *in vivo* that ASM responses to PDGF-BB are consistent with mechanisms of proliferation observed in detail in cultured vascular smooth muscle cells. Specifically, we observe a decrease in gene transcript levels for contractile proteins and ASM hyperplasia associated with changes in lung PDGF-BB levels in airway epithelium and lumen.

Clinical studies investigating PDGF-BB biology have generated conflicting results. Comparison of asthmatic and nasal polyposis biopsies to normal controls demonstrated an elevation of PDGF-BB protein and mRNA in lung eosinophils and epithelial cells(18). In contrast, mRNA and protein expression of PDGF-BB levels were not changed in macrophages from asthmatics compared to stable controls(17,19). When comparing biopsies from asthmatics, COPD patients, and normal smokers, no difference in PDGFR-β were observed(17). No clinical studies that we are aware of have examined the levels of PDGF-BB in asthmatics in both stable and exacerbated states, nor the *activation* of the PDGFR-β, despite reports that PDGFR-β is rapidly upregulated during inflammatory events(27). Furthermore, clinical studies to date have largely focused on PDGF-BB expression from inflammatory and not structural cells. Our chronic allergen exposure data suggests that PDGF-BB is upregulated in lung lavage in times of allergen exposure (24hrs post chronic), but returns to control levels during periods of allergen avoidance (4wks post chronic). Our data supports further analysis of asthmatics pre and post allergen challenge to appropriately determine PDGF-BB biology in more clinically relevant settings.

To date no selective interventions have been developed to disrupt the PDGF-BB signaling pathway, although the promiscuous receptor tyrosine kinase inhibitor imatinib shows some efficacy at blocking the PDGF-R-β, in addition to c-kit, and the intended target the BCR-ABL fusion protein(21). Imatinib has been used in a mouse model of cockroach exposure to inhibit c-kit and has shown efficacy in reducing airway eosinophilia and airway hyperresponsiveness(28). Study outcomes by Berlin et al. did

not include analysis of ASM gene expression, enumeration, or phenotype(28). It remains to be determined if the functional improvements observed with imatinib treatment in this model of allergen exposure was a result of changes in ASM mediated through the PDGF-R-β in addition to the beneficial effects on airway inflammation mediated through c-kit (28)

The clinical efficacy of steroids to treat ongoing airway inflammation in the majority of asthmatics is well described (29). The clinical efficacy of steroids on airway remodeling components, specifically ASM, is less clear, although some animal studies are beginning to address this issue (30). We have incorporated a budesonide treatment arm in our study to investigate whether changes in PDGF-BB biology, gene transcript levels for contractile proteins, and ASM hyperplasia are steroid sensitive. We demonstrate that PDGF-BB is associated with changes in ASM cell phenotype at the gene expression level that are steroid sensitive; More selective interventions are required to strengthen this observation. Additionally, we report that daily budesonide treatment is capable of inhibiting allergen induced changes in lung PDGF-BB biology, gene transcript levels, but not ASM hyperplasia. While these results suggest that PDGF-BB is not required for ASM proliferation, this does not rule out its involvement altogether. It is entirely possible that redundant compensatory mechanisms are involved in ASM hyperplasia including changes in levels of other growth factors (IGF, EGF, bFGF). It is important to emphasize that budesonide treatment and allergen exposure were initiated simultaneously and yet ASM hyperplasia was still observed(30). This finding demonstrates, independent of the efficacy of budesonide on PDGF-BB or other mediator

levels, that steroids may not be any more effective if treatment is initiated at early stages of allergen exposure. Additionally, the removal of allergic stimulus while maintaining budesonide treatment fails to normalize ASM hyperplasia. Clearly, the role of steroids in preventing ASM hyperplasia is questioned by our data, although our data do not address reversal of established ASM hyperplasia.

In the present manuscript we introduce a method for macrodissection of mouse airways that allows for removal of parenchyma, inflammatory infiltrate, and vessels associated with the airway walls. We have previously reported compartment specific (airway wall vs parenchyma) gene expression can be observed using this technique (31). Furthermore we have reported that specific gene signals may not be observed in whole lung homogenate but are observed using dissection methods(32). As vascular smooth muscle expresses similar contractile proteins as ASM, we aimed to remove the former tissue from our preparation to accurately assess gene expression of contractile proteins in ASM alone. Our histology of macrodissected airway demonstrates that airways can be isolated free of associated vasculature while maintaining integrity of airway wall and epithelium (Figure 2).

The studies presented demonstrate several important findings in relation to ASM hyperplasia. We demonstrate for the first time *in vivo*, a PDGF-BB dependent down-regulation of genes encoding contractile proteins and change in cell phenotype with concomitant ASM hyperplasia. Furthermore, chronic allergen exposure results in changes in lung PDGF-BB levels that are associated with reductions in gene transcripts encoding contractile proteins and ASM hyperplasia. While budesonide significantly

attenuated allergen induced changes in lung PDGF-BB levels and gene transcript levels, ASM hyperplasia was resistant to intervention. Changes in ASM phenotype at the gene expression level were not resistant to budesonide treatment. The data suggests that PDGF-BB is sufficient to induce ASM hyperplasia, but alternate pathways may be more relevant in allergen induced increases in ASM content.

Acknowledgments

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Figures

Figure 1: Models of exposure and outcome measurement time-points. **A:** Intratracheal (IT) instillation of $5*10^8$ pfu adenovirus (AdV) PDGF-BB or null (no transgene). Outcome measurements were made on Day 7 following a single AdV instillation . **B:** Allergen sensitization and chronic exposure protocol. Mice were sensitized with intraperitoneal ovalbumin (IP OVA) injections on Days 1 and 11 and intranasal (IN) OVA on Day 11. Chronic allergen exposure was comprised of six 2-day periods of IN OVA administration each separated by 12 days. Exposures started on Days 19 and 20. Control animals received saline instead of OVA. In the chronic allergen exposure model, a separate study arm was performed with daily (S.I.D.) IP injections of budesonide (BUD) 3mg/kg).or vehicle.

Figure 2: Gross morphology and histologic comparison of whole lung and macrodissected isolated airway from mouse lung at low (4X) and high magnification (40X). High magnification performed on area defined by box and extended with dashed lines. Arrows denote airway smooth muscle cells. **A-B:** Whole lung removed **(A)** with histology of large left lobe airway with associated vessels (VL), parenchyma (P), and inflammatory infiltrate (IF). **C-D**: Macrodissected isolated airway tree with vessels and parenchyma removed **(C)** with corresponding histology of large airway. All vessels and parenchyma are removed.

Figure 3: Gene expression analysis on isolated airway preparations from PDGF-BB or null adenovirus treated BALB/c mice expressed as fold change over control **A**: α-SMA. **B**: Smooth muscle myosin heavy chain II (SM-MHC II). **C**: Calponin **D**: Desmin

AdNull – open white bar

AdPDGF-BB – black bar

Data are expressed as mean (SEM); 5 mice per group.

* significantly different from null adenovirus treated animals ($p<0.05$)

Figure 4: Proliferating cell nuclear antigen (PCNA) staining of mouse lung following AdNull **(A-C)** or AdPDGF-BB **(D-F)** instillation. Conventional light microscopy **(A-B, D-E)** or immunofluorescence **(C,F)** at low (10X) and high magnification (40X). High magnification performed on area defined by box and extended with dashed lines. Arrows denote PCNA positive airway smooth muscle cells. Representative images of large airway **(A and B)** and small airways **(D and E).** Fluorescent images are composites of FITC-green (PCNA), TRITC-red (α-SMA), and DAPI-blue (nuclei) channels.

Figure 5: Airway smooth muscle (ASM) quantification following AdNull or AdPDGF-BB instillation (see methods). **A)** Area of colocalized α-SMA and SM-MHC dual stain normalized to basement membrane length. **B)** Number of DAPI nuclei colocalized to α-SMA and SM-MHC dual stain normalized to basement membrane length. **C)** Number of DAPI nuclei colocalized to α-SMA and SM-MHC dual stain normalized to area of α-SMA and SM-MHC dual stain.

AdNull – open white bar

AdPDGF-BB – black bar

Data are expressed as mean (SEM); 5 mice per group.

Figure 6: Airway responsiveness to nebulized methacholine. **A)** Dose response curve. Open squares/solid line: AdNull exposed animals. Black squares/solid line: AdPDGF-BB exposed animals.

* = significantly different from AdNull exposed animals ($p<0.05$).

Data are expressed as mean (SEM); 5 mice per group.

Figure 7: Bronchoalveolar lavage levels of PDGF-BB (pg/ml) following chronic allergen exposure. **A**: 24hrs post chronic allergen exposure. **B**: 4wks post chronic allergen exposure

Saline (SAL) exposure – open white bar

Ovalbumin (OVA) exposure - black bar

OVA + budesonide (BUD) – hatched bar

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from SAL animals ($p<0.05$)

Figure 8: Gene expression analysis on isolated airway preparations following chronic allergen exposure with or without budesonide treatment. **A**: α-SMA. **B**: Smooth muscle myosin heavy chain II (SM-MHC II). **C**: Calponin **D**: Desmin

Naïve + budesonide – open white bar

Saline + vehicle – black bar

OVA + vehicle – 24hrs post exposure – light gray bar

OVA + budesonide – 24hrs post exposure – hatched light gray bar

OVA + vehicle – 4wks post exposure – dark gray bar

OVA + budesonide – 4wks post exposure – hatched dark gray bar

Data are expressed as mean (SEM); 5 mice per group.

* significantly different from saline + vehicle ($p<0.05$)

$ significantly different from OVA + vehicle at same time point ($p<0.05$)

Figure 9: Airway smooth muscle (ASM) cell quantification following chronic allergen exposure with or without budesonide treatment. **A**: 24hrs post chronic exposure. **B**: 4wks post chronic exposure.

Saline + vehicle – open white bar

OVA + vehicle – black bar

OVA + budesonide – hatched bar

Data are expressed as mean (SEM); 10 mice per group.

* significantly different from saline + vehicle ($p<0.05$)

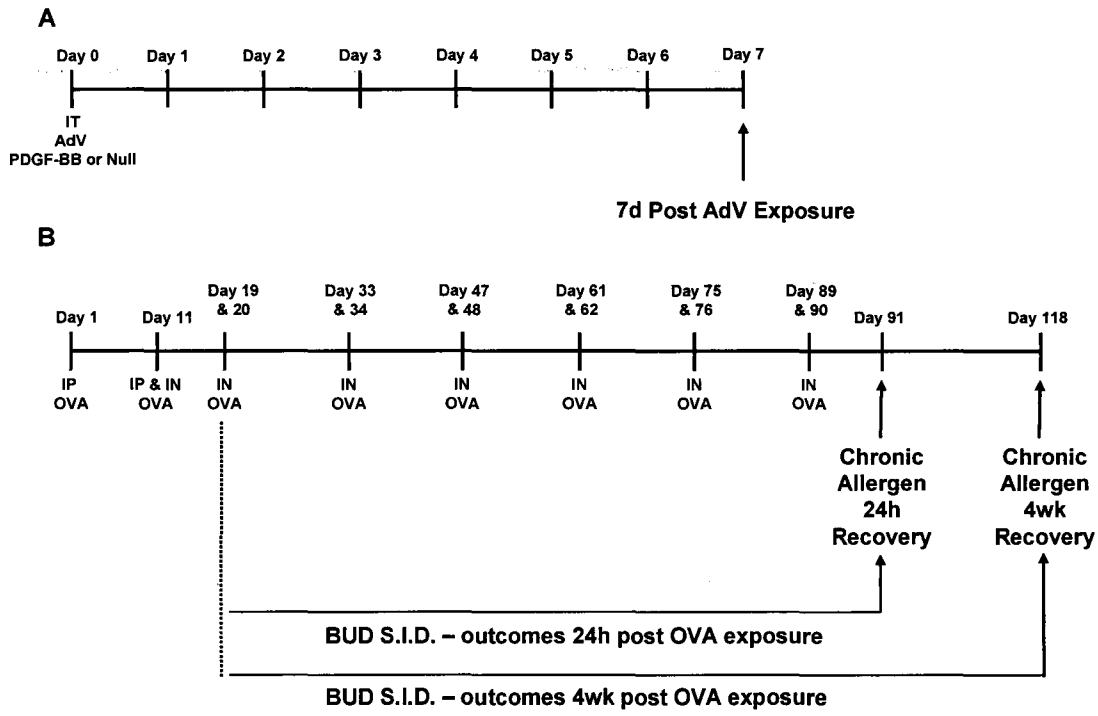
A
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
IT
AdV
PDGF-BB or Null
7d Post AdV Exposure
B
Day 1
Day 11
Day 19 & 20
Day 33 & 34
Day 47 & 48
Day 61 & 62
Day 75 & 76
Day 89 & 90
Day 91
Day 118
IP OVA
IP & IN OVA
IN OVA
Chronic Allergen 24h Recovery
Chronic Allergen 4wk Recovery
BUD S.I.D. – outcomes 24h post OVA exposure
BUD S.I.D. – outcomes 4wk post OVA exposure

Figure 1

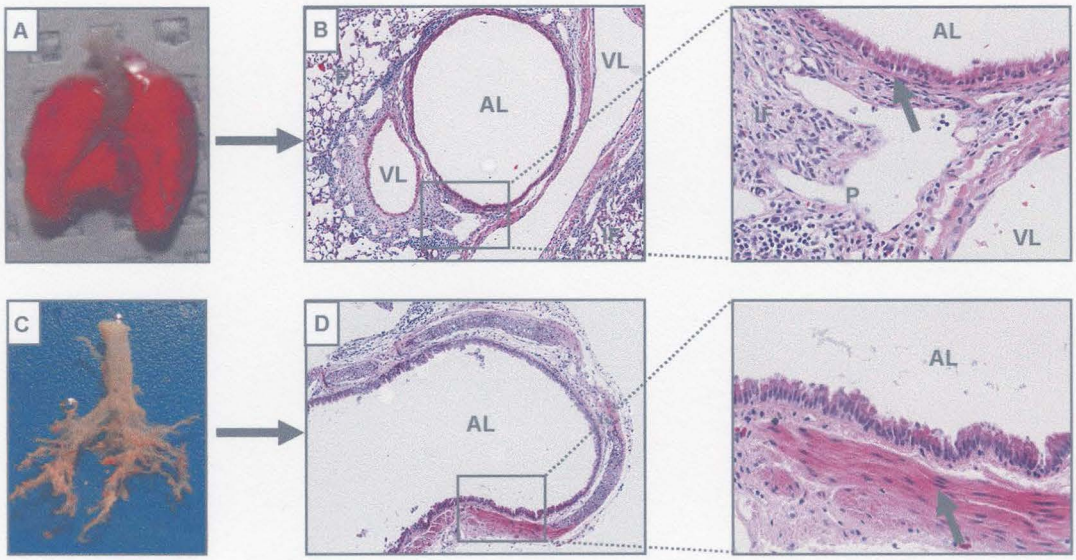

Figure 2

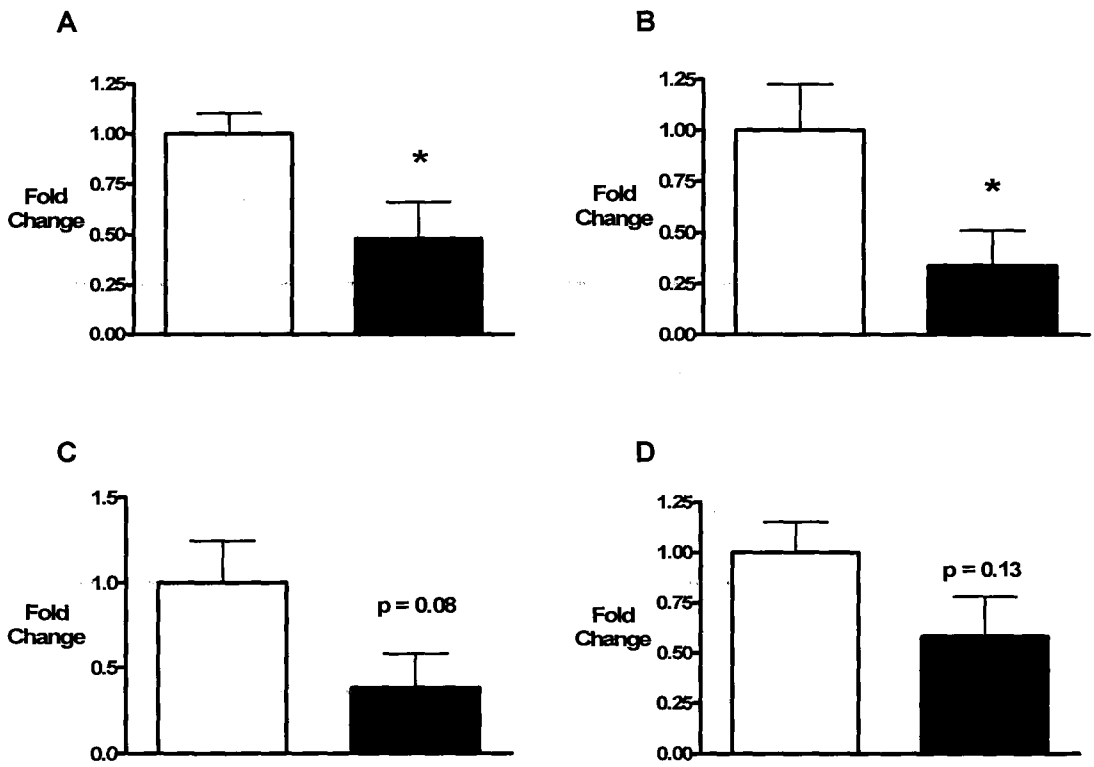

Figure 3

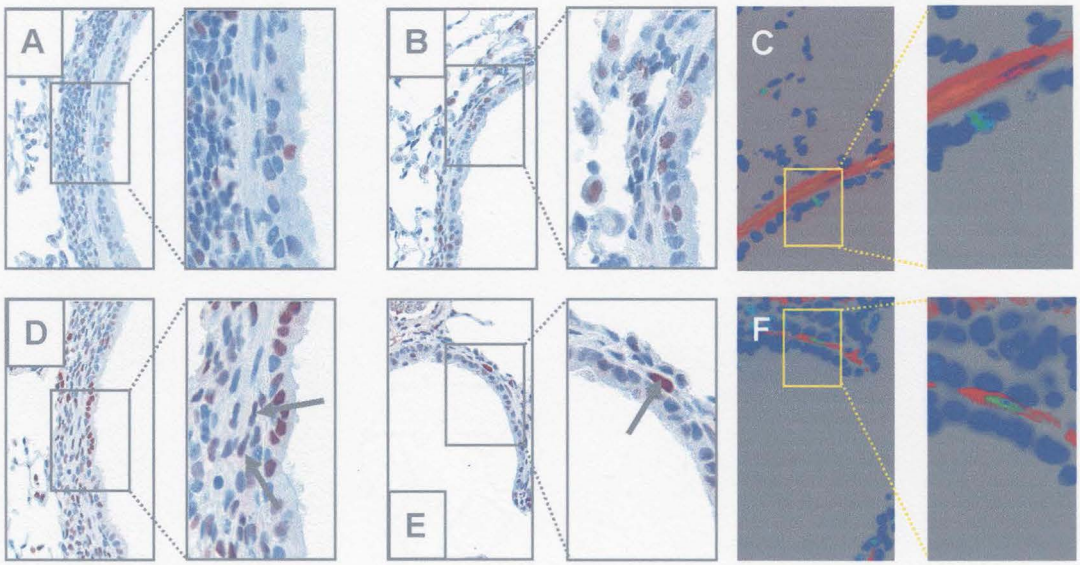

Figure 4

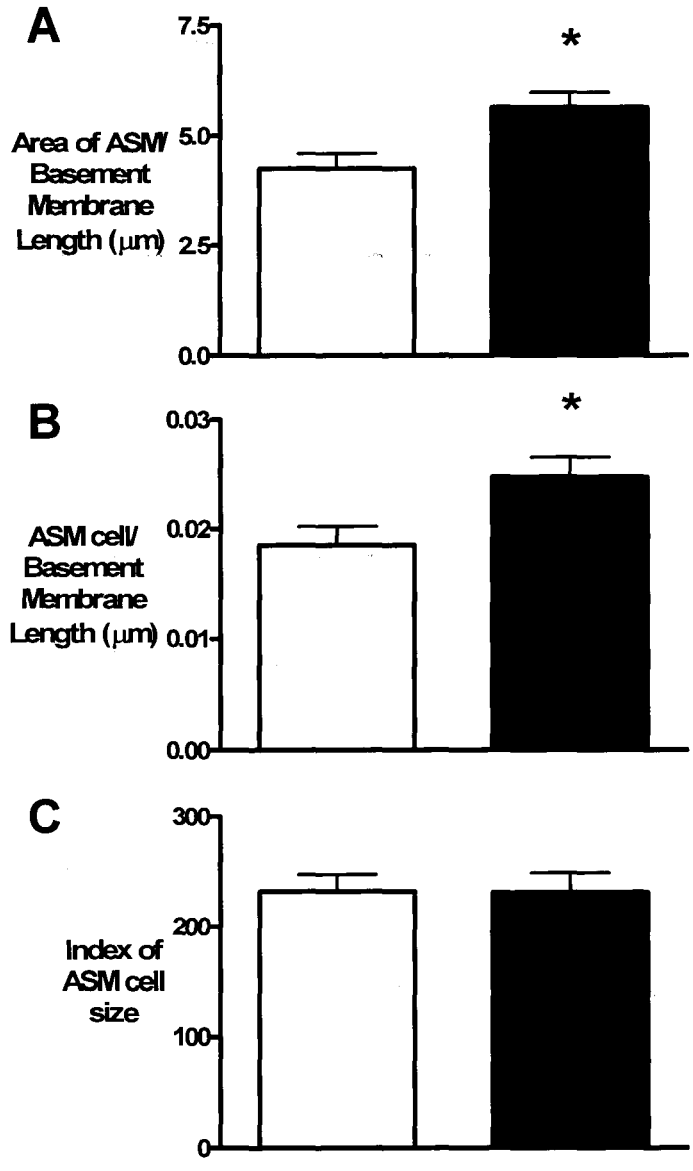
A
7.5
5.0
2.5
0.0
Area of ASM/ Basement Membrane Length (μm)
*
B
0.03
0.02
0.01
0.00
ASM cell/ Basement Membrane Length (μm)
*
C
300
200
100
0
Index of ASM cell size

Figure 5

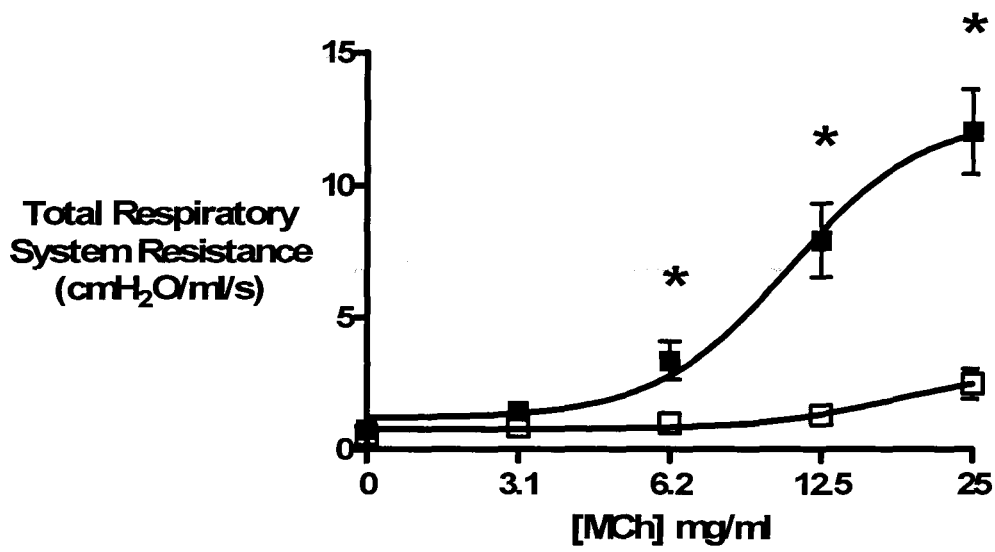
Total Respiratory System Resistance (cmH2O/ml/s)
15
10
5
0
0
3.1
6.2
12.5
25
[MCh] mg/ml
*
*
*

Figure 6

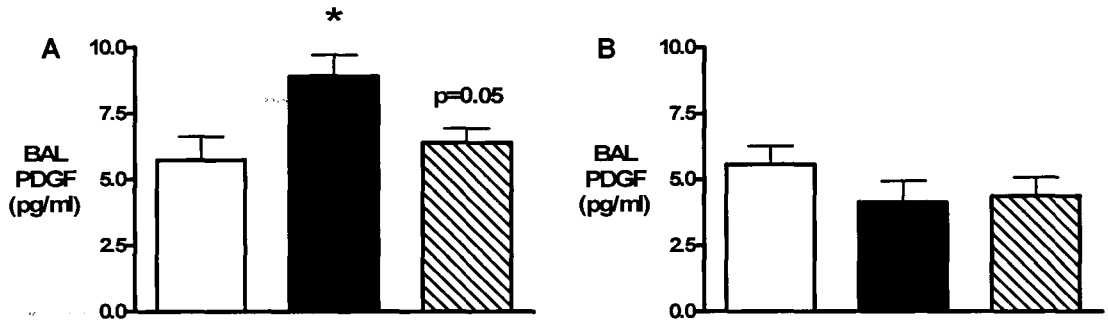
A
10.0
7.5
5.0
2.5
0.0
BAL
PDGF
(pg/ml)
*
p=0.05
B
10.0
7.5
5.0
2.5
0.0
BAL
PDGF
(pg/ml)

Figure 7

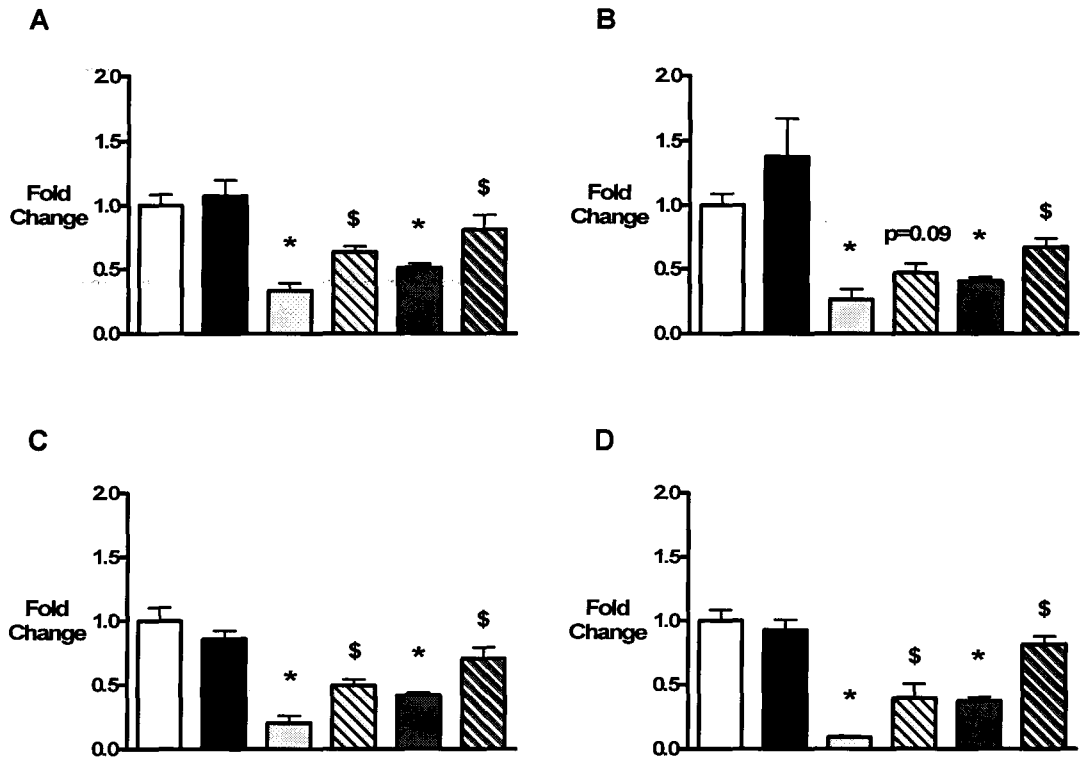

Figure 8

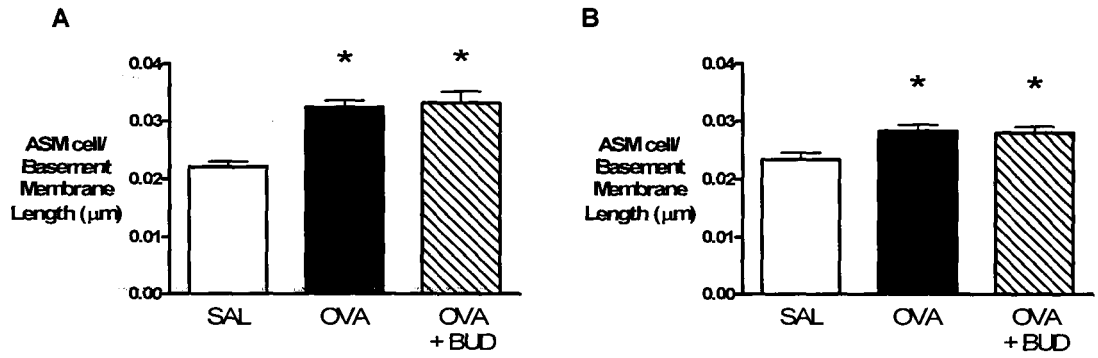
A
B
ASM cell/ Basement Membrane Length (μm)
0.04
0.03
0.02
0.01
0.00
*
*
SAL
OVA
OVA + BUD

Figure 9

CHAPTER 7 : DISCUSSION

Context of Thesis and General Theme

The worldwide burden of asthma is increasing and it is estimated that more than 250000 people die each year from this disease(1). The social and economic costs associated with asthma are vast and are observed in both developed and developing nations. Despite the availability of therapeutics to manage asthma these are unable to prevent development or reverse established asthma(1,2). Clearly there is need for new strategies to improve patient care and disease management aimed at reducing the prevalence of asthma and/or reversing an existing condition. Components of airway remodeling, including thickening of the basement membrane, goblet cell metaplasia, and increased ASM mass, appear to be resistant to conventional therapy and may be an underlying factor limiting the efficacy of current treatment strategies(2-5). Consistent with this view, our general hypothesis for the thesis is that *airway remodeling in response to allergen exposure is a major contributing factor to AHR observed in asthmatics.* We extend our hypothesis by stating that *understanding the mechanisms behind the different components of airway remodeling will provide new avenues for therapeutic development aimed at improving lung function above and beyond current treatment strategies.* To investigate this hypothesis we developed a working theme for the studies contained in the thesis. We set out to use *in vivo* mouse models of exposure to allergen for a) observing the consequences of allergen exposure, b) mechanistically determining biological pathways involved in airway remodeling, and c) developing and testing novel therapeutic strategies. Using this theme, we explore basement membrane thickening(6), goblet cell metaplasia(7), and increased ASM mass(6,8), in our mouse models to generate a greater

understanding of the processes involved in airway remodeling. We anticipate that well characterized animal models with established changes in airway remodeling components will be valuable tools for understanding mechanisms relevant to the human disease and for development and testing of new therapeutics(9).

The following discussion is intended to address the central theme of the thesis rather than a discussion of the individual manuscripts contained within. Discussion of each individual manuscript has been performed already in Chapters 2-6. Instead, an attempt is made to create a discussion that interprets and extends the findings of all manuscripts collectively, highlighting the relationship to the working theme of the thesis.

Heterogeneous Airway Remodeling Processes

Seminal studies demonstrating a thickening in the basement membrane of asthmatics have utilized bronchial biopsies(2,10,11). A limitation of the bronchial biopsy in humans is the number of airway generations that can effectively undergo biopsy(12). The biopsy is performed at the branching of the proximal airways and may not represent the structural environment observed at non-branching regions of the airways. The potential for tissue scarring at the site of biopsy may be a limiting factor in repeated examinations of the airway wall over time(12). Considering this, bronchial biopsies may shed insight into heterogeneous airway remodeling processes but may need to be supplemented by other methods. Circumventing these problems, investigators have examined lungs from fatal asthmatics and control subjects for airway remodeling processes. These anatomical studies on human lungs support the possibility that the

degree of airway wall thickening may vary throughout the airway tree(13), although this study did not specifically look at basement membrane thickness. Our study in mice demonstrates that basement membrane thickening as a result of matrix accumulation may be a heterogeneous process(6). We also highlight that changes in ASM content occur throughout all generations of the airway tree, which may be of relevance to the human disease(14,15).

We can extend our findings from our mouse model to a concept of heterogeneous airway remodeling in humans. The matrix composition of the basement membrane may vary throughout generations in human asthma and this in turn could influence the phenotype of neighbouring ASM cells(16), sequestering of growth factors(17), and airway wall mechanics(18). The mechanisms underlying an increase in ASM proliferation have explored the potential that matrix composition influences ASM phenotype using in vitro culture systems(16). In these studies, the matrix proteins collagen I and fibronectin have been identified as inducers of a proliferative phenotype of ASM. Thus, in generations of the airway tree where the basement membrane expression of collagen I and fibronectin are elevated, a proliferative ASM phenotype could be established leading to increased ASM content. The hypothesis remains to be explored using *in vivo* models and clinical biopsy samples. We could test this hypothesis in our mouse model by using laser capture microdissection techniques to isolate ASM cells from generation of airways that vary in matrix expression profiles and isolating RNA. Gene expression profiling could be used to determine the phenotype of the ASM cells as

has been performed in culture systems(19,20) and identify if *in vivo* changes in proliferative phenotype are related to basement membrane matrix composition.

Our observation that increases in ASM content occur throughout all airway generations is of importance to therapies such as β_2 adrenergic agonists and bronchiothermoplasty that selectively target this tissue. Distribution of β_2 adrenergic agonists and bronchiothermoplasty are limited to the more proximal airways and may have limited impact on the ASM in smaller distal airways(21-23). This may be of considerable importance as asthma has been characterized as a disease of the small airways(14,15,24). Mathematical modeling systems have demonstrated that irrespective of airway diameter (generation), increased ASM mass could be a primary player in airway narrowing(25). If increases in ASM mass are observed in the more distal airways in humans as has been observed in our mice, excessive narrowing could occur at these points where both conventional and unconventional therapies may have reduced efficacy.

Heterogeneous airway remodeling processes may be protective or detrimental to airway function(26) and this may in part depend on the airway generation/localization at which this is occurring. We suggest that an increase in ASM mass in non-cartilaginous airways may have a more pronounced effect on airway function than similar magnitude ASM changes in larger cartilaginous airways due to reduced load. In contrast, an increase in basement membrane thickening in non-cartilaginous airways may actually be protective by increasing ASM load(26). *Ex vivo* experiments examining the contractile properties of different airway sizes in organ baths or lung slice preparations followed by

histological analysis for remodeling components could explore this idea further in mouse model systems or human samples if available(27,28).

We would like to propose an interesting question in context of our observed heterogeneous airway remodeling processes in mice. If airway remodeling processes in the patient population are confirmed to be a heterogenous phenomenon – why are some areas protected while others are not? The difficulty in exploring this question in clinical studies is due to the limitations for retrieval of samples from varying airway generations in the lung. Animal models, and in particular the mouse, may afford investigators the ability to explore these hypothetical processes and provide promising new targets for treatment of airway remodeling.

Mediators and Signalling Pathways Associated with the Allergic Phenotype

Despite the rise in prevalence of asthma throughout the world, there still exists a substantial portion of the population that is *resistant* to developing the disease. The various factors that may influence the development of allergy and asthma include genes(29,30), environment(31), sex(32), temporality of exposure to allergens(33), and infections in childhood(34). It follows that these same factors may also prevent the development of asthma. Our mouse models of exposure to allergen allow for control over many of the aforementioned risk factors. The role of different genetic backgrounds in the development of an allergic asthma-like phenotype was assessed in two genetically distinct inbred strains of mice (BALB/c and C57Bl/6). BALB/c mice developed a robust allergic airway inflammation including elevations in IL-4, IL-13, and eosinophilia that

were associated with airway remodeling processes and AHR(8). Despite allergic inflammation similar in profile to BALB/c mice, C57Bl/6 mice failed to develop overt pathology or changes in airway physiology. Our observed disconnect between the presence of airway inflammatory mediators and pathology may be surprising, although completely interpretable when examining the activity of downstream signalling pathways. We provide supporting evidence showing that a crucial signalling pathway coupled to IL-4 and IL-13 receptors may explain the differences between "prone" and "resistant" mice. By examining the classical signalling pathway downstream of IL-4 and IL-13 we were able to demonstrate a differential regulation and cellular expression profile of STAT6 phosphorylation in BALB/c and C57Bl/6 mice(8). It was apparent that in C57Bl/6 mice the reduced expression of phosphorylated STAT6 was restricted to a cell morphologically distinct from that observed in BALB/c mice and failed to localize to the nucleus. We propose to test the hypothesis in the clinical setting that differential regulation and activation of signalling pathways required for transduction of allergic mediator responses may contribute to development of allergic diseases in humans. The relevance of distinct signalling pathway activities in humans and any relationship with the development of allergy or asthma remains to be determined. Additionally, by demonstrating that elevations in specific inflammatory cells or allergic mediators alone do not confer development or presence of an asthma phenotype, we suggest that any use of these cells or molecules as biomarkers of disease or to monitor management of asthma should be carefully considered and data interpreted accordingly.

Differences in STAT6 phosphorylation could be due to altered ligand/receptor coupling processes(35). Gene polymorphisms between BALB/c and C57Bl/6 mice have been identified for the IL-4Rα chain(36), common to both the IL4 and IL-13 receptor, that may contribute to differential coupling to STAT6. The documented polymorphism in mice or any that may exist in the human population could alter the IL-4 and IL-13 receptor-ligand binding thermodynamics(35) and coupling to STAT6. To date no polymorphisms in the molecules associated with the STAT6 signalling pathway have been reported in the human population. It remains to be determined if differences in coupling of key allergic mediators to signal transduction pathways may be differentially regulated between asthmatics and non-asthmatics.

It is entirely possible that the receptor or signalling molecules required for propagating IL-4 and IL-13 messages are not different between our BALB/c "prone" and C57Bl/6 "resistant" mice or in asthmatics and non-asthmatics. Changes in the regulatory mechanisms governing the lifecycle of STAT6(37), including nuclear import mechanisms, degradation, and re-shuttling, may exist and influence development of an asthmatic phenotype(37). The mechanisms responsible for STAT6 nuclear import are currently unknown and may represent a therapeutic target aimed at inhibiting IL-4 and IL-13 signaling(38,39). Suppressors of cytokine signaling (SOCS-1) or opposing molecular signaling pathways (STAT1) could constitute alternate methods for regulating the STAT6 lifecycle(40). The recent development and pre-clinical testing of a cell penetrating inhibitory peptide for STAT6 may prove useful in altering the STAT6 life-cycle, although the mechanism of action for this compound is not completely

understood(41). Perhaps differences in endogenous mechanisms regulating STAT6 life cycle exist in the population and are related to manifestation of allergic diseases including asthma and allergic rhinitis. Interpretations from our findings and new inhibitors(41) suggest that a better understanding of the regulatory mechanisms of the STAT6 signaling pathway may yet uncover novel therapeutic targets.

Mechanisms of Goblet Cell Metaplasia

Goblet cell metaplasia may result in excessive mucus production and plugging of airways, is associated with fatal asthma attacks(42). Observations of goblet cells and/or increased mucus production in airways are not limited to asthma and are observed in chronic obstructive pulmonary disease and cystic fibrosis patients(43,44). As the mechanisms underlying goblet cell metaplasia and mucus hypersecretion in each airway disease may share a common pathway, understanding the mechanisms involved in these processes in asthma may provide benefit in multiple airway diseases. Previously published reports have demonstrated the central role of IL-13 in the development of goblet cell metaplasia in asthma models(45,46). We and others have provided experimental data from mouse models supporting the role of IL-13(47), although the precise mechanisms that orchestrate the change of airway epithelial cells to mucus producing and secreting goblet are only now being elucidated(48). In this thesis we present data that characterize an IL-13 dependent, epithelial GABAergic signalling mechanism, that contributes to goblet cell metaplasia(7). We further characterize the mechanism by using a selective IL-4Rα monoclonal antibody to inhibit the classical IL-

13 signaling pathway. Our results suggest that upstream signalling pathways that lead to GABAergic upregulation in airway epithelium may prove useful for the development of intervention strategies for managing goblet cell metaplasia(49).

Our studies explored the use of two distinct classes of molecules, different from conventional β_2 adrenergic agonists and glucocorticoids, to treat the asthma phenotype. In the seminal study describing the GABAergic signalling pathway in airway epithelium, the use of GABA antagonists were effective in inhibiting mucus production in a model of exposure of allergen to mice(7). The selective $GABA_A$ receptor antagonists, picrotoxin and bicuculline, may provide a management strategy for goblet cell metaplasia when delivered topically. Clinical studies would need to be performed to test this hypothesis and investigate any potential side effects associated with inhibitory GABAergic actions in the CNS. Our characterization of the GABAergic processes in airway epithelium following brief house dust mite exposure was consistent with previous findings observed in OVA and recombinant IL-13 models. In our brief house dust mite model we utilized a monoclonal antibody to selectively inhibit the IL-4Rα and explore the signaling pathways involved in IL-13 mediated upregulation of the GABAergic system. Our data demonstrates the efficacy of this class of molecules in treating allergic responses and is consistent with the clinical use of Omalizumab, a monoclonal antibody against IgE(50). We suggest that both GABA antagonists and monoclonal antibodies targeting molecules associated with goblet cell metaplasia may allow for management of asthma above and beyond current level of control.

The Role of PDGF-BB in ASM Proliferation

Increases in ASM mass are routinely observed in the airways of asthmatics(13,51,52). The functional consequences of increased ASM mass have been mathematically modeled and may contribute substantially to AHR(18,25). Recent non-pharmacologic interventions aimed at selective ablation of ASM have demonstrated improvement in clinical outcomes, supporting this tissue as a suitable target(21,53). Unfortunately the mechanisms responsible for increased ASM in asthmatic airways remain poorly understood. It is postulated that increases in ASM mass may be the result of several processes including hypertrophy(51), hyperplasia(52), reduced rates of apoptosis(54), and migration from peripheral(55) or systemic regions(56).

Cell culture models of ASM have been integral into investigating the growth of ASM under a variety of conditions. Proliferation in response to growth factors (PDGF-BB, IGF, bFGF) has been studied in detail *in vitro*, but these studies have not been extended to *in vivo* model systems(19,57,58). Using the mouse, we demonstrate the ability of PDGF-BB to induce ASM proliferation which was associated with AHR. Changes in gene expression patterns consistent with a proliferative phenotype of ASM were also observed(20). We did not assess markers of apoptosis and therefore can not rule out that PDGF-BB provides a pro-survival signal in addition to mediating proliferation. The extension of our studies into a model of chronic allergen exposure demonstrated a hyperplastic phenotype of ASM associated with elevations in levels of PDGF-BB in lung. The lack of efficacy of glucocorticoids in treatment of airway remodeling components has been addressed in clinical biopsy studies(2) examining

airway wall thickening, but not ASM specifically. A budesonide treatment arm in our study was unable to prevent development of ASM hyperplasia, highlighting the need for development of therapeutics aimed to selectively target ASM. These treatments may be effective above and beyond what current therapeutic and management strategies afford patients.

The clinical studies that have examined PDGF-BB biology in the context of asthma have been observational in nature and have used asthmatics that are under disease control(59-62). The changes in PDGF-BB levels in lung inflammatory and structural cells have not been explored in the context of a controlled allergen challenge setting. Until experiments in humans have been performed during periods of exacerbation the clinical relevance of changes in PDGF-BB levels, the activation of its rapidly upregulated receptor(63), and any downstream consequences on ASM proliferation remain to be determined.

Study Limitations

Medical research routinely employs experimental model systems to investigate disease processes as experimental access to humans is limited due to ethics and resource availability. The model systems used are by default deviations from the human disease and each has their own strengths and weaknesses. When interpreting results from any experimental model one must consider these strengths and weaknesses to arrive at appropriate conclusions.

As demonstrated in this thesis, our lab routinely uses mouse models of exposure to allergen to model asthma. In our introduction we highlighted the many degrees of freedom that exist in allergen exposure mouse model development(9). The variables to be considered could be levels of experimental control, but they also can limit the extrapolations of the results to human asthma. The homogeneous genetic makeup of each highly inbred mouse strain demonstrates a drastic level of control that on one hand is beneficial, while on the other hand is not representative of genetically diverse human population.

It is important to emphasize that mice do not naturally develop an asthma phenotype (elevated IgE, eosinophilia, AHR). Artificial allergen sensitization methods are required to produce the asthma phenotype and therefore may not accurately model the processes involved in human sensitization to antigen(64).

Allergen induced changes in airway physiology in mice are substantially smaller in magnitude as compared to changes in airway function observed in the clinic. Despite our ability to model significant allergen induced increases in airway responsiveness to nebulized methacholine in mice, changes in indices of airway responsiveness remain only 1.5-5 times greater than control animals. Clinical assessment of airway responsiveness demonstrates a wide range of responses in asthmatic PC_{20} values that can vary by 10000 times(65).

The invasive methods for determining lung mechanics in mice, which require intubation, ventilation, and anesthesia, are drastically different than what is performed in the clinic. The clinical relevance of airway function measurements in a ventilated,

anesthetized, intubated animal that has been administered neuromuscular blocking agents (pancuronium) to prevent voluntary breathing has been questioned(66,67). To address these limitations, modified methods of whole body plethysmography using orthogonal video cameras to estimate changes in lung volume while monitoring changes in pressure have been validated to assess airways resistance in unrestrained animals(67). These methods have yet to be validated using animals varying in lung compliance, such as our allergen exposure mice. The further development and validation of methods of unrestrained assessment of airway resistance may provide an alternative to invasive measurements that are far removed from clinical practice.

The physiology and anatomy of the mouse is clearly different from humans. Mice are obligate nasal breathers with a lung architecture that has fewer branches than human lungs(68). Distribution of inhaled allergen or bronchoconstrictor during measurements of airway physiology in mice may be different than in humans due to a monopodial branching respiratory system (as compared to dichotomous in humans)(64). The absence of bronchial circulation in the airway of mice may limit the ability to model and investigate allergen induced effects of edema. In humans, edema may alter the structure of the basement membrane and contribute to airway dysfunction(69). In mice, the basement membrane is substantially thinner and due to the lack of bronchial circulation may not undergo changes possible in humans. Additionally, the thinner basement membrane is difficult to differentiate into lamina propria and reticularis using conventional histology methods. Another significant difference in the anatomy of the mouse lung is the absence of mucus glands and ducts. The role of mucus glands in

asthma and their impact on any airway function can therefore not be investigated using allergic mouse models.

The results obtained and presented in this thesis are focused on mouse model systems. Our studies lack the translation of the mouse model findings to clinical studies. Experimenting with clinical samples to further characterize the biological processes observed in our mouse models would add significant strength to our findings.

Future Directions and Translation to Clinical Medicine

Medical research is an ongoing process that will always exist to challenge existing dogma in attempts to improve the quality of life of humans. From small observational basic research studies to large multi-centred clinical trials conducted on international scales, there always remain avenues to pursue further to generate knowledge. It is the view of this author that some of the most important studies to be performed are observational – as they plant the seeds for future experiments aimed at understanding a given observation in greater detail using mechanistic approaches.

In Chapter 2 we characterize the development of airway remodeling indices in a mouse model of chronic exposure to allergen. Our findings support a concept of heterogeneous airway remodeling processes that may be of relevance to humans. Although biopsies are limited in their ability to analyze varying airway generations, they may be combined with modern clinical imaging techniques (70) to test the null hypothesis that airway remodeling is homogeneous. Clinical data in support of the null hypothesis would suggest that therapeutics designed to target airway remodeling

processes would be effective throughout all airway generations. The consequences of rejecting the null hypothesis would require investigators to determine why some generations or specific areas within a given generation of airway are "prone" while others are "resistant". Furthermore, imaging methods combined with segmental bronchoprovocation tests could explore the functional consequences of hetergenous airway remodeling processes(70).

The translation of mouse modeling data to clinical studies is of central interest to this author. Although highly robust, our observations in Chapter 3 of distinct staining patterns and expression levels of signalling molecules associated with the allergic response may be a mouse phenomenon(8). We demonstrate that STAT6 phosphorylation levels and staining pattern occurs in distinct inflammatory cells in an allergic, airway remodeling "prone" mouse strain, while a different expression profile is observed in "resistant" mice. We would like to test the hypothesis that expression patterns and levels of STAT6 phosphorylation are related to the manifestation of allergic diseases. Clinical biopsy samples and sputum cell smears from pre-post allergen challenged allergic asthmatics, allergic non-asthmatics, and controls could be examined for STAT6 phosphorylation levels and staining patterns to test this hypothesis. Currently it is unknown if polymorphisms of STAT6 or regulators of this molecule exist amongst the general population that could support our hypothesis. Clearly therapeutics aimed at interfering with STAT6 could be pursued further in both animal models and clinical studies to determine the relevance of this molecule in the disease process. Reducing

STAT6 availability, decreasing rates of phosphorylation, inhibiting nuclear import mechanisms all could be explored as avenues for therapeutic development.

The testing and development of new therapeutics and their targets in mouse models form the foundations for pursuit into clinical studies. Current asthma therapeutics are not effective in managing mucus overproduction, a leading cause of deaths in asthma(42). In Chapters 4 and 5 we identify and characterize a mechanism involved in mucus overproduction in epithelial cells. To date several GABA antagonists (bicucculine, picrotoxin) have been identified and could be tested for efficacy in managing goblet cell metaplasia and excessive mucus production in asthma(71). Although, our studies focused on models and clinical samples of asthma, the results could also be explored in other mucus hypersecretion airway diseases and models including chronic obstructive pulmonary disease(44). Additionally, our pre-clinical evaluation of monoclonal antibodies developed against specific molecules involved in mucus overproduction highlight the use of this class of compounds in asthma therapy. The application of anti-IL-4Rα monoclonal antibodies to treat *established* mucus over production in chronic mouse models of allergen exposure will provide information on the ability of these compounds to reverse a disease process.

The role of ASM in the asthmatic lung is currently being investigated by selective ablation using an invasive technology called bronchothermoplasty(21,53). A recent editorial has highlighted the benefits of this technique but also the need to explore the mechanisms by which this procedure works to reduce ASM content(72). Important to emphasize, we do not understand the mechanisms by which an increase in ASM mass

occurs in the airways of asthmatics. We demonstrate in Chapter 6 a mechanism for ASM hyperplasia that should be explored further in clinical studies. During periods of allergen exposure an elevation in PDGF-BB is observed in mice. Further characterization of this model should be performed by analyzing receptor activity and localization to determine the relevance of this signaling pathway to ASM hyperplasia. Although PDGF-BB and it's receptor expression have been explored in asthmatics, this has been primarily been in inflammatory cells and during periods of asthma control(59-62). Investigating growth factors, including PDGF-BB, that are upregulated in response to controlled allergen challenge in an experimental clinic setting would provide evidence to support targeting these factors. Small molecule inhibitors of receptor tyrosine kinases already exist and may be useful in targeting the effects of growth factors. Indeed, the promiscuous tyrosine kinase inhibitor imatinib, used to inhibit the bcr-abl fusion protein responsible for chronic myeloid leukemia, also has antagonist activity at the PDGF-β receptor(73). The clinical testing of imatinib in asthmatic patients may treat both airway remodeling components (through blocking of PDGF-β) and inflammation (through blocking c-kit). These studies remain to be performed.

Final Conclusion

The worldwide burden of asthma is on the rise despite the availability of therapeutics used to manage the disease. Airway remodeling processes that result in fundamental changes to the structure of the airway wall are resistant to current

therapeutics. We hypothesize that it is the airway remodeling components that contribute to sustained AHR observed in asthmatic patients(3).

Asthma is a heterogenous disease amongst the population, and it is possible that heterogeneity of disease exists within a given patient. By observing and characterizing a mouse model of chronic allergen exposure we provide data that supports heterogeneous airway remodeling processes throughout different airway generations. The clinical consequences of this remain to be determined. In addition to observing and characterizing our models, we explore the possibility of new therapeutic compounds and strategies. Consistent with this, the findings presented in this thesis support the pursuit of cell penetrating peptides to interfere with intracellular signalling pathways(41), $GABA_A$ antagonists(7), and monoclonal antibodies to relevant antigens for the treatment of airway remodeling components in human asthma. Understanding the biological processes of airway remodeling that are currently resistant to therapeutics should provide insight into new targets. To date the mechanisms responsible for *in vivo* ASM mass increases are unknown. We have explored these mechanisms and highlight the role that PDGF-BB can play in ASM hyperplasia. Development of therapeutics aimed at growth factors and their receptors may provide an approach to the management of airway remodeling processes observed in asthma, previously unexplored. The studies presented in this thesis promoted a greater characterization of *in vivo* mouse models of allergen exposure combined with the application of these models to mechanistically explore the biological pathways involved in the different components of airway remodeling. We have demonstrated that these models are effective tools for the testing of therapeutic strategies targeting airway

remodeling pathways of interest and anticipate that they will remain useful in future research.

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