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THE UNCOUPLING OF THE INFLAMMATORY AND STRUCTURAL COMPONENTS OF AIRWAY HYPERRESPONSIVENESS

By

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Richard Leigh

Airway hyperresponsivess (AHR) is a characteristic feature of asthma. However, the mechanisms underlying AHR are complex and likely to be multi-factorial. A number of animal models have focused on understanding the mechanisms of *transient* airway inflammation and the associated *transient* airway dysfunction that occur following brief allergen exposure. While much has been learned about the role of acute inflammation in this brief allergen-induced response, a limitation of these studies is that the airway dysfunction is not fully representative of the AHR present in asthma. For example, we have shown that effective anti-inflammatory treatment results in only a modest reduction in AHR, indicating that AHR is usually sustained in asthma. This suggests that other mechanisms, including airway remodeling, likely play an important role in the pathophysiology of AHR. The subsequent focus of this thesis has been firstly, to demonstrate that sustained airway dysfunction develops in mice chronically exposed to allergen, and secondly, to explore the potential mechanisms and associations underlying this phenomenon. By subjecting sensitized mice to either brief or chronic periods of allergen exposure, we have developed a novel model in which chronic allergen exposure results in sustained airway dysfunction and structural changes of the airway. Subsequently, mice deficient for IL-4, IL-5 or IL-13 were studied using similar protocols. IL-4 and IL-13, but not IL-5, are critical for the development of airway remodeling, and in the absence of remodeling, mice were protected from developing sustained airway dysfunction. In further experiments, mice were either T cell immuno-depleted using

monoclonal antibodies, or were treated with an anti-IL-13 fusion protein. When these interventions were given *after* chronic allergen exposure, at a time when airway remodeling was established, neither intervention attenuated the *sustained* airway dysfunction. Taken together, these results provide new information about basic physiological mechanisms underlying the sustained airway dysfunction present in asthma.

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Chapter 1

INTRODUCTION

Asthma is characterized by paroxysmal symptoms such as breathlessness, chest tightness, wheezing, sputum production and cough (1). These symptoms are the result of episodic bronchoconstriction and variable airflow limitation that occur secondary to an underlying dysfunction of the airway, termed airway hyperresponsiveness (AHR) (2;3). Generally, *airway responsiveness* is the preferred term used to describe the physiological ability of the airways to narrow after exposure to a variety of chemical and physical bronchoconstrictor agonists. Thus, airway hyperresponsiveness is characterized by exaggerated airway narrowing in response to a variety of non-specific physical, chemical or pharmacological stimuli that have little or no effect in healthy subjects (4-6). These include physical stimuli such as hyperventilation of cold, dry air as occurs with exercise (7), chemical mediators such as cysteinyl leukotrienes (8), and pharmacological bronchoconstricting agonists, such as methacholine, histamine and adenosine (2;9;10). AHR is present in almost all individuals with asthma, particularly in those in whom symptoms of asthma are present at the time of evaluation (5). However, the molecular and cellular mechanisms underlying this physiological phenomenon have not yet been fully elucidated (6:11). The manuscripts that make up this thesis provide substantial novel information about the basic physiological mechanisms that underpin AHR, and the purpose of this introductory chapter is to provide an understanding of the background to this research by reviewing the current knowledge of the basic mechanisms that underlie AHR.

Historical Perspective

Episodic airway dysfunction has long been recognized to be a characteristic feature of asthma. In a seminal paper in 1859, Henry Hyde-Salter recognized the presence of variable airflow limitation in asthma with the following description, "paroxysmal dyspnoea of a peculiar character, generally periodic, with intervals of healthy respiration between attacks." (12). In 1921, Alexander and Paddock demonstrated symptoms of variable airflow limitation occurred more readily in asthmatic individuals compared to healthy subjects, following subcutaneous administration of the cholinergic constricting agonist pilocarpine (13). These initial observations were later corroborated by Weiss and colleagues in 1932, when they reported that asthmatic subjects, but not healthy subjects, developed bronchoconstriction (measured by changes in vital capacity) after being given intravenous histamine (14). In the mid 1940's, Tiffeneau and colleagues (15) and Curry (16) introduced the concept of AHR, when they provided descriptions of direct bronchial challenge testing using threshold doses of inhaled acetylcholine, histamine or methacholine to determine the degree of airway responsiveness in asthmatic individuals. Curry's report was also the first to demonstrate that that the magnitude of the bronchocontrictor response in asthmatic subjects following inhaled histamine and methacholine was related to the severity of disease (16). Subsequent working definitions of asthma emerged from two Ciba-Geigy Foundation symposia in 1959 (17) and 1971 (18), which emphasized that variable airflow limitation was the fundamental abnormality underlying asthma. These symposia also highlighted the importance of AHR as a characteristic feature of asthma, and pointed to the need for

the development of simple, generally applied methods for measuring the phenomenon. Subsequently, standardized protocols were established to measure AHR in response to inhaled bronchoconstrictor mediators (2;19;20) and measurements of AHR, using inhalation challenges with airway constrictor agonists such as histamine or methacholine, have now become routine practice in the diagnosis and assessment of patients with asthma (5;21). Current working definitions of asthma represent modifications of the original Ciba definition, and continue to recognize AHR as a defining characteristic of asthma (1;22;23). It is therefore not surprising that much of the experimental and clinical research exploring the underlying pathogenesis of asthma has focused on better understanding the exact mechanisms underlying AHR.

Airway Dysfunction in Asthma

AHR can be demonstrated in almost all patients with current symptomatic asthma. (2;11). The measurement of AHR involves the patient inhaling increasing concentrations (or doses) of a constricting agent, until a given level of bronchoconstriction (typically a 20% fall in forced expired volume in 1 second (FEV₁)) is achieved. Airway responsiveness can then be quantified as the provocative concentration, or dose, of that agent required to produce a 20% fall in FEV₁, and this measurement is referred to as the PC₂₀ or PD₂₀ respectively (5). Using the method described by Cockcroft and colleagues (2), asthmatic subjects generally have a provocative concentration of histamine or methacholine causing a 20% fall in FEV₁ (PC₂₀) of <8 mg/mL. Most non-asthmatic patients will have a PC₂₀ of >16 mg/mL; however, defining the exact level of airway

responsiveness which would categorically distinguish asthmatic and non-asthmatic individuals is not possible. This is because there appears to be a continuous unimodal log-normal distribution of non-specific airway reponsiveness in the general population, with patients with asthma representing the *hyperresponsive* part of the distribution curve (24-26).

The physiological dysfunction underlying AHR in asthma includes; 1/ hypersensitivity of the airways to a constrictor agonist, as indicated by a smaller concentration of the agonist needed to initiate the bronchoconstrictor response (leftward shift of the bronchoconstrictor dose-response curve); 2/ hyperreactivity of the airways to a constrictor agonist (increased slope of the dose response curve) and 3/ a greater maximum degree of induced bronchoconstriction (maximal degree of respiratory resistance) to the agonist (9). In normal individuals, the dose response curve is characterized by modest functional responses that plateau at high doses of agonist. In contrast, the curves from patients with varying severities of asthma (mild, moderate and severe AHR) are typically different with respect to airway sensitivity (in asthma curves are shifted to the left), airway reactivity (increasing asthma severity is associated with steeper slopes) and the maximal degree of bronchoconstriction (often not measurable in patients with moderate to severe AHR). An illustration of these responses is given in Figure 1. Although an increase in the slope of the dose-response curve (airway reactivity) or in maximum bronchoconstrictor response may be observed, these individual variables are typically not reported when assessing airway responsiveness in individual subjects; instead, the concentration of the bronchoconstrictor required to produce a 20% fall in FEV_1 (PC₂₀) is calculated as a single variable. The term *airway hyperresponsiveness* is used to describe a PC₂₀ or PD₂₀ lower than the normal range (i.e. PC₂₀ methacholine <16mg/ml), and may reflect one or more of the specific abnormalities described above (11;27).

Comparisons between patients, as well as variations *within* individual patients, can be quantified as doubling concentration differences in PC_{20} (21). The difference in airway responsiveness between normal subjects and patients with asthma is substantial, with differences of 4-10 doubling concentrations in PC_{20} methacholine often present between normal and asthmatic individuals (5). Furthermore, while AHR was initially considered a static property of the airways, it has become increasingly clear that it is a dynamic process that can vary over time. Thus, while most, if not all asthmatics have some degree of AHR, its magnitude is not fixed within asthmatic individuals but rather appears to fluctuate with the current asthma severity (2;28-31). Furthermore, a number of studies have now consistently demonstrated that exacerbations of asthma, whether occurring naturally as in seasonal allergen exposure (32;33), viral infection (34), or as a result of experimental allergen exposure (21;35;36), or following the withdrawal of corticosteroid treatment (37;38) are associated with a worsening of PC₂₀ by 1-2 doubling concentrations within individual patients. In contrast, improvement in asthma severity, as occurs for example following allergen avoidance (39;40) or treatment with inhaled corticosteroids (41-44) is usually associated with an improvement in PC20 by 1-2 doubling concentrations within individual patients. The magnitude of these modulating effects on airway responsiveness is illustrated in Figure 2.

Mechanisms of AHR in Asthma

Over the last 30 years, progress has been made regarding our understanding of the biology of asthma, and more specifically, of the mechanisms involved in the pathophysiology of AHR (6;11;45;46). This has been due, in part, to the progress made in the techniques used to investigate asthma; original investigation relied on the use of postmortem samples, and subsequent investigations were restricted to the measurement of airflow limitation and inflammatory cell numbers in peripheral blood. It was not until the advent of the fibre-optic bronchoscope that the detailed pathology of asthma and AHR was advanced in a systematic manner. Concurrent advances in morphometric, cellular, and molecular techniques have allowed detailed analyses of bronchial biopsy specimens, and enabled detailed description of the inflammatory and morphological changes present in airways of asthmatic individuals (45-54). Currently it is thought that both immune-mediated airway inflammation as well as chronic structural changes of the airway wall (often described under the collective term of *airway remodeling*), are important in the development of AHR (6;11). However, the exact contributions of these processes to the pathophysiology of AHR remain to be fully elucidated.

Role of Reversible Inflammatory Processes

Immune-mediated airway inflammation, in which CD4⁺ and CD8⁺ T lymphocytes orchestrate a predominantly T-helper type 2 (Th2) response characterized by the release of mediators such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-4, IL-5 and IL-13, as well as by the infiltration of activated mast cells, basophils, eosinophils and monocytes, is a characteristic feature of asthma (45;46;53;55-

57). Early reports suggested that the extent of eosinophilic airway inflammation was related to the degree of AHR (58;59), and the view that the eosinophil was central to the pathogenesis of AHR was supported by both *in-vitro* and experimental evidence that eosinophil granule proteins caused contraction of airway smooth muscle, and also caused significant increases in airway reactivity when administered directly to the airways of non-human primates (60-62). Subsequently, a number of studies have suggested possible relationships between other inflammatory cell types, including activated T lymphocytes, mast cells, basophils and mononuclear cells (56;63-68), as well as between specific inflammatory mediators such as the cysteinyl leukotrienes (8;69) and AHR. However, while the concept that one or more cells or mediators may cause AHR is attractive, the exact contribution of airway inflammation to the pathophysiology of AHR remains relatively poorly defined (11;21;27;70-72).

The role of immune-mediated airway inflammation in the pathogenesis of AHR has been studied in great detail in clinical models of asthma, particularly allergen-induced asthma. Different forms of allergen-exposure, such as challenge with a single dose of allergen (73-75), exposure to repeated low doses of allergen (36;76), or natural seasonal exposure to a pollen allergen (33) all increase airway inflammation, as judged by biopsy specimens, lavage samples, or induced sputum examination. These studies have thus provided insight into how changes in AHR are regulated by induced inflammatory processes, and support the paradigm that acute inflammatory responses, and airway eosinophilia in particular, contribute to the severity of AHR. However, they also demonstrate that while acute exposure to allergen results in marked increases in airway

eosinophilia, it is generally produces a decrease (worsening) in PC_{20} methacholine of only 1 to 2 doubling concentrations, which represents a change of much smaller magnitude than those seen when asthmatic patients with persistent AHR are compared to healthy subjects, in whom the differences are in the range of 4 to 8 doubling doses (21;77-79) (**Figure 2**). These allergen-induced changes are however clinically important, as it is likely that the mechanisms responsible for the changes in AHR following experimental allergen exposure are similar to those producing transient worsening of asthma control during exacerbations of the disease.

Further insight into the role of immune-mediated airway inflammation in the pathogenesis of AHR has been provided by studies evaluating the effects of allergen avoidance or anti-inflammatory treatments in asthma. Eliminating or decreasing allergen load by allergen avoidance improves, but does not eliminate AHR (40;80;81). Similarly, A number of studies have reported that inhaled corticosteroid treatment, in doses and for durations sufficient to minimize indices of acute immune-mediated airway inflammation, has only minor effects on attenuating the degree of AHR present in asthmatic subjects (41;44;82-86). Indeed, even 2 years of treatment with high doses of inhaled corticosteroids did not produce much more than a doubling concentration improvement in PC_{20} (43). The results of these studies have led to the hypothesis that there may be ongoing inflammatory mechanisms that are important in the pathophysiology of AHR, which are relatively insensitive to the anti-inflammatory effects of corticosteroids. Cysteinyl leukotrienes have been shown to be important in the immunopathology of asthma (87-89) and their inflammatory effects may not be inhibited by corticosteroid

treatment (90). Hamilton and colleagues (91) have evaluated the effects of pretreatment with pranlukast, a leukotriene receptor antagonist, on allergen-induced AHR. However, while treatment resulted in significant attenuation of allergen-induced early and late asthmatic responses, it only afforded protection of approximately 1-2 doubling concentrations against allergen-induced AHR, and did not result in abrogation of the AHR into the normal range (**Figure 2**).

It seems unlikely therefore, based on these and other treatment studies, that acute inflammatory events in the airway account for all of the aspects of the AHR seen in asthmatic subjects, who often have PC_{20} methacholine measurements as much as 500 fold below normal. In addition, a number of studies have now been published showing weak or no relationship between eosinophilic airway inflammation and degree of AHR (72;92-95). Rather than being viewed as negative findings, these observations should be viewed as evidence that the mechanisms of AHR in asthma are complex, and that finding a strong correlation with a single pathologic process is unlikely to occur. Instead, the frequently observed lack of association between airway inflammation and AHR makes it increasingly clear that other factors are likely to be involved in the pathophysiology of this condition. Based on the studies discussed above, it appears that the pathophysiology underlying AHR is the result of two temporally independent processes, namely 1/a variable, responsive component, which can be attributed to fluctuating degrees of airway inflammation, and 2/a sustained component that remains relatively fixed, suggesting it is independent of acute inflammatory events within the airway (Figure 2).

Role of Airway Remodeling

While the factors responsible for the sustained component of AHR in asthma are not well characterized, recent literature has implicated airway remodeling as a contributing factor in the development and persistence of AHR (53;54;96-102). Although changes in the structure of the airway wall have been recognized as a feature of asthma for over 80 years (103;104), the term *airway remodeling* appears to have been coined by Lynne Reid, first in 1966 with regard to lung development (105), and later again in 1978 in her J. Burns Amberson Lecture on the structural changes that occur in the pulmonary vasculature during pulmonary hypertension (106). However, the concept of airway remodeling in asthma was only proposed in 1992 (107), and can be defined as changes in the composition, content and organization of the cellular and molecular constituents of the airway wall (53;96;102;108;109). Early pathological investigation relied on postmortem samples, and several necropsy study reports indicated that the airways of asthmatic individuals dying from acute severe asthma demonstrated chronic structural changes, when compared to airways of non-asthmatic individuals dying of non-natural causes (110-117). While these reports implied that chronic structural changes are secondary phenomena, developing late in the disease process of severe asthma, subsequent analysis afforded by flexible fibreoptic bronchoscopy and bronchial biopsy demonstrated that structural changes are also present in the airways of individuals with symptomatically mild asthma (47;48;50;51;118-122). These structural changes include; thickening and disruption of the epithelium with associated epithelial and goblet cell hyperplasia; sub-epithelial fibrosis; transformation of fibroblasts to myofibroblasts;

thickening of the inner airway wall between the airway smooth muscle layer and the subepithelial region with an associated increase in the number and size of vessels within this region; smooth muscle hypertrophy and hyperplasia; and thickening of the adventitia as a result of extracellular matrix deposition (47;63;110;111;115;117;119;123-131). However, despite advances in our understanding of the inflammatory and immunological components of asthma, there is relatively little understanding of the cellular and molecular mechanisms that underpin the chronic structural changes present in asthmatic airways, and even less understanding of how these changes might relate to the pathophysiology of AHR.

Is remodeling a result of chronic inflammation?

Although the exact pathogenesis of airway remodeling has not been fully elucidated, there is considerable evidence that immune mediated airway inflammation provides an environment that is conducive to airway remodeling. Observations that the extent of the fibrotic changes present are related to the degree of airway eosinophilia (132), and that these fibrotic changes are increased in patients with allergic rhinitis who later develop asthma (86) support the concept that at least some of the remodeling changes are progressive in response to an underlying immune-mediated inflammatory process. Asthma is characterized by a Th2-type inflammatory response in the airways, and the cytokines IL-4 and IL-13 have both been implicated in the pathogenesis of airway remodeling (133). IL-4 and IL-13 are known to be fibrogenic and have direct effects on both epithelial cells and fibroblasts (134-136). By interacting with cells within the

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epithelial-mesenchymal trophic unit, IL-4 and IL-13 appear to be able to augment chronic inflammation and airway remodeling. Other growth factors that are increased in the asthmatic airway include transforming growth factor (TGF)- β (137-140), platelet derived growth factor (PDGF)- β , (141;142), IL-1 β (143) as well as altered ratios of matrix metalloproteinases (MMP) to tissue inhibitors of MMPs (TIMPs) (144;145). These, and other mediators, have been associated with deposition of collagen and extracellular matrix, growth and proliferation of smooth muscle, myofibroblast differentiation from fibroblasts and epithelial cells, as well as goblet cell and epithelial hyperplasia (146-149).

A number of models in which animals are sensitized to an antigen, such that Th2 type inflammatory responses occur upon subsequent airway challenge, have also supported the concept that asthmatic type airway inflammation is able to promote airway remodeling. Several laboratories have demonstrated that repeated allergen challenge results in epithelial hypertrophy in rats, guinea pigs and mice (150-153); goblet cell hyperplasia in rats and mice (151;154); subepithelial fibrosis in rats and mice (150;151;153;154); and increases in smooth muscle staining in rats and guinea pigs (150-152;155). While these studies do not confirm that remodeling occurs in asthma as a result of repeated Th2 inflammatory events, they illustrate that mechanisms necessary for this to occur do exist, and are consistent with the hypothesis that such inflammatory events may be a causal mechanism by which airway remodeling occurs.

Does remodeling contribute to AHR?

Much of our understanding regarding the functional effects of airway remodeling is based on theoretical mathematical modeling studies of the human airway (102). This is partly because clinical studies evaluating the functional consequences of airway remodeling have been hampered by the difficulty in obtaining sufficient numbers of tissues from asthmatic patients to make a statistically valid comparison with tissues from normal subjects. These modeling studies predict that thickening of the airway wall, which can occur as a result of epithelial or goblet cell hyperplasia, subepithelial fibrosis or local edema, is able to amplify the effect of airway smooth muscle shortening, and is thus likely to be a major contributor to AHR. Moreno and colleagues (156) modeled the effect of thickening of the inner airway wall to show how it can amplify the degree of luminal narrowing for a given degree of airway smooth muscle shortening. They reported that increasing thickness of the inner airway wall produced simulated agonist response curves that closely resembled in vivo airway challenges in asthmatic individuals, at least with respect to the increase in maximal airway narrowing. This mathematical model has since been used in more realistic computational schema, incorporating measured values for airway wall dimensions from asthmatic and normal airways, and these studies have confirmed the original predictions of Moreno's study (115;157-160). Theoretical modeling studies also predict that increases in airway smooth muscle mass result in an increased force of contraction and an enhanced ability of the airways to generate radial stress and airway narrowing (158). Thus, increases in airway smooth muscle mass, which is a consistent pathological finding in asthma (110;117;122) is likely to be a major

determinant of AHR. This hypothesis is, however, based on the unproven assumption that the proliferated smooth muscle maintains the contractile properties of normal airway smooth muscle; the precise role of increased airway smooth muscle mass in the development of AHR in vivo is still uncertain. Finally, modeling studies also suggest that subepithelial fibrosis may increase the extent of airway narrowing by interfering with the development of mucosal folds (161). It is believed that the airway mucosa folds when airway smooth muscle shortens. For geometrical reasons, it is expected that the lower the number of folds, the greater the reduction in airway lumen (162). Thus, the possibility that collagen deposition beneath the layer of smooth muscle may enhance the contractility of the airways is in keeping with the model of Wiggs, et al. (157), in which increases in airway wall thickness by sub-epithelial collagen deposition results in enhanced airway responses to bronchoconstrictor stimuli, for the same degree of smooth muscle constriction. However, theoretical predictions of mucosal folding and its effects are heavily influenced by model assumptions (162-164), and no proof has been provided on how mucosal folding occurs in vivo and whether this is different between health and disease. Thus, although many of these modeling studies have evaluated aspects of remodeling in isolation, they nonetheless provide convincing evidence that multiple structural changes associated with airway remodeling are likely to contribute to the pathophysiology of AHR.

A number of clinical studies have correlated the extent of subepithelial fibrosis or smooth muscle thickness with either the degree of AHR (93;165), or with asthma symptoms (120). In addition, several studies have shown that indices of airway

inflammation and chronic structural changes consistent with airway remodeling are less pronounced in subjects with asymptomatic AHR than in patients with documented asthma (126;166). Furthermore, the subsequent development of symptoms of asthma was associated with an increase in airway inflammation and indices of airway remodeling, which correlated with a further increase in AHR (166). However, given the complex and multi-factorial nature of the factors contributing to AHR, observations from clinical studies such as these are likely to only provide evidence of weak relationships between potential causes and functional effects; they are unlikely to provide substantial insights into the mechanisms underlying AHR. To progress beyond this level of understanding would ideally require patients to be followed from a time-point before airway remodeling begins, and then longitudinally as the remodeling process advances, so that effects on airway function could be described. However, a recent biopsy study in young children (167) reported that indices of airway remodeling were often present before the onset of asthma symptoms, suggesting that this process likely begins early in the pathogenesis of asthma. Thus, longitudinal clinical studies with regular biopsy sampling of airway tissues are not logistically or ethically feasible; instead, this is an ideal scenario in which to use experimental animal models to test scientific hypotheses and gain further insight into the mechanisms underlying AHR.

The Contribution of Mouse Models to the Understanding of AHR

A number of animal models have been developed to investigate the mechanisms underlying AHR. An advantage of animal models in general is they allow for *in vivo*

manipulation of certain outcome variables, and in vitro study of easily accessible cells and tissues using approaches that are often technically or ethically not feasible to perform in human subjects (168). Mouse models, in particular, have additional advantages in that there is minimal genetic variation within in-bred strains thereby providing appropriate experimental control animals; there are also large numbers of well-characterized gene or receptor deletion (knockout) strains available, which express deficiencies of numerous individual inflammatory cells or cytokines. This has resulted in a great number of studies that have characterized immuno-pathological mechanisms involved in allergic airway inflammation. However, to extend these experimental observations into appropriate models of allergen-induced AHR, it was necessary to demonstrate that the induction of immune-mediated airway inflammation resulted in physiological airway dysfunction, similar to that seen in asthma. As a result, a number of physiological techniques have been developed to assess airway function in mice. To facilitate understanding of the techniques used, it is useful to consider the many available in vivo techniques in mice in two broad categories; 1) indirect measurements that are thought to be related to airway function, or 2) direct measurements of airway impedance. Indirect measurement techniques include the widely used technique of measuring airway function by placing an unrestrained and conscious mouse into an essentially airtight chamber, and then measuring the pressure fluctuations within the chamber, by barometric whole body plethysmography (Buxco, Troy, NY) (169). A relationship, termed enhanced pause (Penh), which is based on the magnitude and timing of the pressure fluctuation within the whole body plethysmograph, has been correlated with the magnitude of respiratory

system resistance and elastance and shown to be responsive to inhaled constrictor agonists and exposure to allergen. **Direct** measurement techniques share in common the measurement of *airflow* or *lung volume* changes, and the driving pressures responsible for achieving both flow and lung volume changes. In all cases the measured flow is integrated with respect to time to calculate volume change, or the measured volume change is differentiated with respect to time to calculate flow. Direct methods are generally regarded as superior by those investigators interested in the components responsible for physiological airway dysfunction following allergic airway inflammation. Indirect methods, being technically easier to perform, are generally preferred by investigators whose focus of research is on the immunological consequences of allergen exposure. In our laboratory, a method has been established that is considered to provide a *direct* assessment of airway impedance. A detailed explanation of this method is provided in the *Appendix*.

With the advent of appropriate techniques to measure airway physiology, considerable attention has been paid to mouse models of allergen-induced airway inflammation and the associated airway dysfunction, in an attempt to further elucidate the potential mechanisms underlying allergen-induced AHR (170-186). While an extensive review of this literature is not appropriate in this chapter, there have been at least 100 similar studies investigating the mechanisms of allergen-induced AHR in mice. Virtually all these models involve sensitization followed by airway challenge, with a resulting transient period of lymphocytic and eosinophilic airway inflammation and associated *transient* airway dysfunction (187). These studies have also given rise to some debate

over which of the recognized Th2 cytokines are responsible for acute allergen-induced eosinophilic inflammation and airway dysfunction. Reports on the effects of IL-5 have consistently demonstrated that IL-5 is required for acute allergen-induced eosinophilic airway inflammation, and that the extent of eosinophilic inflammation is markedly reduced in the absence of IL-5 (173;188-190); however most investigators have been unable to demonstrate that either IL-5 deficiency or blockade has an effect on AHR (173;180;181;185;188;191). A second consistent finding is that IL-4 is not required for the development of acute allergen-induced eosinophilic airway inflammation (173;180;188;191). An initial report suggesting that IL-4 was required for the development of AHR (192) was not supported by subsequent publications (180;185;188;191). More recently, several investigators have proposed a critical role for IL-13 in the pathophysiology of allergen induced airway dysfunction, in that IL-13 was able to induce airway dysfunction when delivered into mouse airways in the absence of allergen (178;179), or when IL-13 over-expressed in transgenic mice (136). Initial reports suggested that blocking IL-13 alone was sufficient to prevent AHR (178;179), but it has recently been suggested that additional IL-4 blockade is required for this manipulation to be maximally effective (185). These murine models have however focused on reproducing the acute inflammatory events and the associated transient airway dysfunction that occur following brief exposure to allergen. Despite the advances in our understanding provided by these studies, a major limitation of the majority of these models is that the airway dysfunction is transient, disappearing 14-21 days after allergen exposure, and appears to be related only to acute increases in immune mediators and

inflammatory cells (187). This transient AHR is not equivalent to the sustained AHR present in subjects with asthma, and that is likely to account for most of the differences, in terms of doubling doses of bronchoconstricting agonist, between subjects with and without asthma. Thus, while studies such as these have provided valuable information on the mechanisms responsible for acute responses to allergen, they are unlikely to provide a complete description of the complex and likely multi-factorial mechanisms responsible for sustained AHR. As a result, a major research focus in our laboratory has been to investigate the mechanisms underlying this sustained AHR, and the potential role that chronic, inflammatory-induced structural changes of the airway may contribute to these processes. In addition to our own laboratory, a number of other laboratories have also developed a keen interest in determining the physiologic effects of induced airway remodeling in animal models of chronic allergen challenge (151;154;193-200). In most of the models already described, investigators have been able to demonstrate that animals were hyperresponsive to non-specific bronchoconstrictors following a period of chronic allergen challenge. However, the results from these studies are difficult to interpret, in that these measurements were all made shortly after the final allergen challenge. Clearly, based on models of brief exposure to allergen, there are transient immune-mediated effects on airway responses at this time. Thus, assessment of the *independent* contribution of airway remodeling to AHR will require studying animals at a later time point, some weeks after final allergen exposure, when transient immune responses are likely to have subsided.

Purpose of this Doctoral Thesis

The overall objective of this thesis has been to investigate the mechanisms underlying AHR, and the potential role that airway remodeling may contribute to this process, by means of a series of well-focused research projects. In the initial clinical study (Chapter 2), we evaluated the effects of an inhaled corticosteroid (budesonide) and a leukotriene antagonist (montelukast) that, in combination, were theoretically able to abrogate the inflammatory pathways responsible for both the early and late asthmatic responses in an allergen-challenge model. Our results indicate that, despite significantly attenuating both the early and late phase responses as well as preventing any significant allergen-induced sputum eosinophilia, study subjects only experienced a modest 1-2 doubling dose protection in allergen-induced AHR. These results confirm that while acute, immune-mediated airway inflammation may contribute to a variable responsive component of AHR, it is unlikely to account for the sustained component of AHR that remains relatively fixed despite appropriate anti-inflammatory therapy. This observed lack of association between acute airway inflammation and AHR makes it increasingly clear that other factors are likely to be involved in the pathophysiology of this condition. The remaining 5 papers contained in the thesis describe, 1/ the initial development of an experimental model, in which mice subjected to chronic periods of allergen exposure develop sustained airway hyperreactivity and indices of airway remodeling compared to mice subjected to brief allergen exposure (Chapter 3); 2/ the validation of the morphometric techniques used in the mouse studies (Chapter 4); 3/ the role of the Th2 cytokines IL-4, IL-13 and IL-5 in the pathogenesis of sustained AHR (Chapter 5); 4/

airway remodeling as a potential mechanism responsible for *sustained* AHR, by demonstrating that blocking acute, immune-mediated inflammatory responses at a time when chronic structural changes have developed, has no effect on *sustained* AHR (*Chapters 6 & 7*). The clinical implications of these studies, and the novel contributions that they provide to our understanding of the pathophysiology of a common clinical problem, are discussed in *Chapter 8*.

<u>References</u>

- Boulet LP, Becker A, Berube D, Beveridge R, and Ernst P: Canadian Asthma Consensus Report, 1999. Canadian Asthma Consensus Group. CMAJ 1999, 161: S1-S61
- 2. Cockcroft DW, Killian DN, Mellon JJA, and Hargreave FE: Bronchial reactivity to inhaled histamine: a method and clinical survey. Clin Allergy 1977, 7: 235-243
- 3. Xu X, Rijcken B, Schouten JP, and Weiss ST: Airways responsiveness and development and remission of chronic respiratory symptoms in adults. Lancet 1997, 350: 1431-1434
- 4. Sterk PJ: Bronchial hyperresponsiveness: definition and terminology. Pediatr Allergy Immunol 1996, 7: 7-9
- Cockcroft DW: Airway Responsiveness. Asthma. Edited by Barnes PJ, Grunstein MM, Leff AR, and Woolcock AJ. Philadelphia, Lippencourt-Raven Publishers, 1997, pp. 1253-1266
- 6. Brusasco V and Pellegrino R: Complexity of factors modulating airway narrowing in vivo: relevance to assessment of airway hyperresponsiveness. J Appl Physiol 2003, 95: 1305-1313
- 7. O'Byrne PM, Ryan G, Morris M, McCormack D, Jones NL, Morse JL, and Hargreave FE: Asthma induced by cold air and its relation to nonspecific bronchial responsiveness to methacholine. Am Rev Respir Dis 1982, 125: 281-285
- 8. Adelroth E, Morris MM, Hargreave FE, and O'Byrne PM: Airway responsiveness to leukotrienes C4 and D4 and to methacholine in patients with asthma and normal controls. N Engl J Med 1986, 315: 480-484
- 9. Woolcock AJ, Salome CM, and Yan K: The shape of the dose-response curve to histamine in asthmatic and normal subjects. Am Rev Respir Dis 1984, 130: 71-75

- Holgate ST, Mann JS, and Cushley MJ: Adenosine as a bronchoconstrictor mediator in asthma and its antagonism by methylxanthines. J Allergy Clin Immunol 1984, 74: 302-306
- 11. O'Byrne PM and Inman MD: Airway hyperresponsiveness. Chest 2003, 123: 411S-416S
- Salter HH: The pathology of asthma its absolute nature. Classic papers in asthma. Edited by Brewis RAL. London, Science Press Limited, 1990, pp. 106-142
- 13. Alexander HL and Paddock R: Bronchial asthma: response to pilocarpine and ephedrine. Arch Intern Med 1921, 27: 184-191
- 14. Weiss S, Robb GP, and Ellis LP: The systemic effects of histamine in man. Arch Intern Med 1932, 49: 360-396
- 15. Tiffeneau R and Beauvallet R: Epreuve de bronchoconstriction et de bronchodilation par aerosols. Bull Acad Med 1945, 129: 165-168
- 16. Curry JJ: The action of histamine on the respiratory tract of normal and asthmatic subjects. J Clin Invest 1946, 25: 785-791
- 17. Ciba Foundation Guest Symposium. 1959. Terminology, definitions, and classifications of chronic pulmonary emphysema and related conditions. Thorax 1959, 14: 286-299
- 18. Report of the Working Group on the Definition of Asthma. Ciba Found Study Group 1971, 38: 172-174
- 19. Chai H, Farr RS, Froehlich LA, Mathison DA, McLean JA, Rosenthal RR, Sheffer AL, Spector SL, and Townley RG: Standardization of bronchial inhalation challenge procedures. J Allergy Clin Immunol 1975, 56: 323-327

- 20. Shapiro GG, Simon RA, and Sinon RA: Bronchoprovocation committee report. American Academy of Allergy and Immunology. J Allergy Clin Immunol 1992, 89: 775-778
- 21. Inman MD, Hamilton AL, Kerstjens HA, Watson RM, and O'Byrne PM: The utility of methacholine airway responsiveness measurements in evaluating antiasthma drugs. J Allergy Clin Immunol 1998, 101: 342-348
- 22. Guidelines on the management of asthma. Statement by the British Thoracic Society, the Brit. Paediatric Association, the Research Unit of the Royal College of Physicians of London, the King's Fund Centre, the National Asthma Campaign, the Royal College of General Practitioners, the General Practitioners in Asthma Group, the Brit. Assoc. of Accident and Emergency Medicine, and the Brit. Paediatric Respiratory Group. Thorax 1993, 48: S1-24
- 23. New NHLBI guidelines for the diagnosis and management of asthma. National Heart, Lung and Blood Institute. Lippincott Health Promot Lett 1997, 2: 1, 8-1, 9
- 24. Cockcroft DW, Berscheid BA, and Murdock KY: Unimodal distribution of bronchial responsiveness to inhaled histamine in a random human population. Chest 1983, 83: 751-754
- 25. Woolcock AJ, Peat JK, Salome CM, Yan K, Anderson SD, Schoeffel RE, McCowage G, and Killalea T: Prevalence of bronchial hyperresponsiveness and asthma in a rural adult population. Thorax 1987, 42: 361-368
- 26. Boulet LP: Asymptomatic airway hyperresponsiveness: a curiosity or an opportunity to prevent asthma? Am J Respir Crit Care Med 2003, 167: 371-378
- 27. Lotvall J, Inman M, and O'Byrne P: Measurement of airway hyperresponsiveness: new considerations. Thorax 1998, 53: 419-424
- 28. Boushey HA, Holtzman MJ, Sheller JR, and Nadel JA: Bronchial hyperreactivity. Am Rev Respir Dis 1980, 121: 389-413

- 29. Juniper EF, Frith PA, and Hargreave FE: Airway responsiveness to histamine and methacholine: relationship to minimum treatment to control symptoms of asthma. Thorax 1981, 36: 575-579
- 30. Cartier A, Thomson NC, Frith PA, Roberts R, and Hargreave FE: Allergeninduced increase in bronchial responsiveness to histamine: relationship to the late asthmatic response and change in airway caliber. J Allergy Clin Immunol 1982, 70: 170-177
- 31. Ryan G, Latimer KM, Dolovich J, and Hargreave FE: Bronchial responsiveness to histamine: relationship to diurnal variation of peak flow rate, improvement after bronchodilator, and airway calibre. Thorax 1982, 37: 423-429
- 32. Boulet LP, Cartier A, Thomson NC, Roberts RS, Dolovich J, and Hargreave FE: Asthma and increases in nonallergic bronchial responsiveness from seasonal pollen exposure. J Allergy Clin Immunol 1983, 71: 399-406
- 33. Djukanovic R, Feather I, Gratziou C, Walls A, Peroni D, Bradding P, Judd M, Howarth PH, and Holgate ST: Effect of natural allergen exposure during the grass pollen season on airways inflammatory cells and asthma symptoms. Thorax 1996, 51: 575-581
- 34. Pizzichini MM, Pizzichini E, Efthimiadis A, Chauhan AJ, Johnston SL, Hussack P, Mahony J, Dolovich J, and Hargreave FE: Asthma and natural colds. Inflammatory indices in induced sputum: a feasibility study. Am J Respir Crit Care Med 1998, 158: 1178-1184
- 35. Gauvreau GM, Doctor J, Watson RM, Jordana M, and O'Byrne PM: Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. Am J Respir Crit Care Med 1996, 154: 1267-1271
- 36. Sulakvelidze I, Inman MD, Rerecich T, and O'Byrne PM: Increases in airway eosinophils and interleukin-5 with minimal bronchoconstriction during repeated low-dose allergen challenge in atopic asthmatics. Eur Respir J 1998, 11: 821-827
- 37. Gibson PG, Wong BJO, Hepperle MJE, Kline PA, Girgis-Gabardo A, Guyatt G, Dolovich J, Denburg JA, Ramsdale EH, and Hargreave FE: A research method to induce and examine a mild exacerbation of asthma by withdrawal of inhaled corticosteroid. Clin Exp Allergy 1992, 22: 525-532
- 38. in 't Veen JCCM, Smits HH, Hiemstra PS, Zwinderman AH, Sterk PJ, and Bel EH: Lung function and sputum characteristics of patients with severe asthma during an induced exacerbation by double-blind steroid withdrawal. Am J Respir Crit Care Med 1999, 160: 93-99
- 39. Benckhuijsen J, van den Bos JW, van Velzen E, de Bruijn R, and Aalbers R: Differences in the effect of allergen avoidance on bronchial hyperresponsiveness as measured by methacholine, adenosine 5'-monophosphate, and exercise in asthmatic children. Pediatr Pulmonol 1996, 22: 147-153
- 40. van Velzen E, van den Bos JW, Benckhuijsen JA, van Essel T, de Bruijn R, and Aalbers R: Effect of allergen avoidance at high altitude on direct and indirect bronchial hyperresponsiveness and markers of inflammation in children with allergic asthma. Thorax 1996, 51: 582-584
- 41. Woolcock AJ, Yan K, and Salome CM: Effect of therapy on bronchial hyperresponsiveness in the long-term management of asthma. Clin Allergy 1988, 18: 165-176
- 42. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, and Johansson SA: Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. Am Rev Respir Dis 1992, 145: 890-899
- 43. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, and Sterk PJ: Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am J Respir Crit Care Med 1999, 159: 1043-1051
- 44. Inman MD, Watson RM, Rerecich T, Gauvreau GM, Lutsky BN, Stryszak P, and O'Byrne PM: Dose-dependent effects of inhaled mometasone furoate on airway function and inflammation after allergen inhalation challenge. Am J Respir Crit Care Med 2001, 164: 569-574

- 45. Bousquet J, Chanez P, and Lacoste: Eosinophilic inflammation in asthma. N Engl J Med 1990, 323: 1033-1039
- 46. Busse WW and Lemanske RF, Jr.: Asthma. N Engl J Med 2001, 344: 350-362
- 47. Laitinen LA, Heino M, Laitinen A, Kava T, and Haahtela T: Damage of the airway epithelium and bronchial reactivity in patients with asthma. Am Rev Respir Dis 1985, 131: 599-606
- 48. Haahtela T, Laitinen A, and Laitinen LA: Using biopsies in the monitoring of inflammation in asthmatic patients. Allergy 1993, 48: 65-69
- 49. Laitinen A and Laitinen LA: Pathology of asthma. Allergy Proc 1994, 15: 323-328
- 50. Laitinen LA, Laitinen A, Altraja A, Virtanen I, Kampe M, Simonsson BG, Karlsson SE, Hakansson L, Venge P, and Sillastu H: Bronchial biopsy findings in intermittent or "early" asthma. J Allergy Clin Immunol 1996, 98: S3-S6
- 51. Laitinen A, Laitinen LA, and Virtanen IT: Bronchial biopsies. Asthma. Edited by Barnes PJ, Grunstein MM, Leff AR, and Woolcock AJ. Philadelphia, Lippincott-Raven, 1997, pp. 209-223
- 52. Laitinen A, Karjalainen EM, Altraja A, and Laitinen LA: Histopathologic features of early and progressive asthma. J Allergy Clin Immunol 2000, 105: S509-S513
- 53. Bousquet J, Jeffery PK, Busse WB, Johnson M, and Vignola AM: Asthma. From bronchoconstriction to airways inflammation and remodeling. Am J Respir Crit Care Med 2000, 161: 1720-1745
- 54. Vignola AM, Kips J, and Bousquet J: Tissue remodeling as a feature of persistent asthma. J Allergy Clin Immunol 2000, 105: 1041-1053
- 55. Howarth PH, Wilson J, Djukanovic R, Wilson S, Britten K, Walls A, Roche WR, and Holgate ST: Airway inflammation and atopic asthma: a comparative bronchoscopic investigation. Int Arch Allergy Appl Immunol 1991, 94: 266-269

- 56. Bradley B, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AA, Schwartz LB, Durham SR, Jeffery PK, and Kay AB: Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: Comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. J Allergy Clin Immunol 1991, 88: 661-674
- 57. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan CJ, Durham SR, and Kay AB: Evidence for a predominant "Th-2-type" bronchoalveolar lavage T-lymphocyte population in atopic asthma. N Eng J Med 1992, 326: 298-304
- 58. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, and Kay AB: Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. Am Rev Respir Dis 1988, 137: 62-69
- 59. Brusasco V, Crimi E, Gianiorio P, Lantero S, and Rossi GA: Allergen-induced increase in airway responsiveness and inflammation in mild asthma. J Appl Physiol 1990, 69: 2209-2214
- 60. Gundel RH, Letts LG, and Gleich GJ: Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. J Clin Invest 1991, 87: 1470-1473
- 61. Coyle AJ, Mitzner W, and Irvin CG: Cationic proteins alter smooth muscle function by an epithelium-dependent mechanism. J Appl Physiol 1993, 74: 1761-1768
- 62. Uchida DA, Ackerman SJ, Coyle AJ, Larsen GL, Weller PF, Freed J, and Irvin CG: The effect of human eosinophil granule major basic protein on airway responsiveness in the rat in vivo. A comparison with polycations. Am Rev Respir Dis 1993, 147: 982-988
- 63. Beasley R, Roche WR, Roberts JA, and Holgate ST: Cellular events in the bronchi in mild asthma and after bronchial provocation. Am Rev Respir Dis 1989, 139: 806-817

- 64. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, and Holgate ST: Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. Am Rev Respir Dis 1990, 142: 863-871
- 65. Robinson DS, Bentley AM, Hartnell A, Kay AB, and Durham SR: Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. Thorax 1993, 48: 26-32
- 66. Holgate S: Mediator and cytokine mechanisms in asthma. Thorax 1993, 48: 103-109
- Frew, A. J. and Djukanovic, R. Inflammatory Mechanisms in Asthma. Holgate, S. T. and Busse, W. B. 1st[117], 27-38. 1998. New York, Marcel Dekker Inc. Lung Biology in Health and Disease. Lenfant, C.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, and Pavord ID: Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med 2002, 346: 1699-1705
- 69. Laitinen LA, Laitinen A, and Haahtela T: Leukotriene E₄ causes granulocyte infiltration into asthmatic airways. Lancet 1993, 341: 989-990
- 70. O'Byrne PM, Hargreave FE, and Kirby JG: Airway inflammation and hyperresponsiveness. Am Rev Respir Dis 1987, 136: S35-S37
- 71. Brusasco V, Crimi E, and Pellegrino R: Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. Thorax 1998, 53: 992-998
- 72. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, and Brusasco V: Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. Am J Respir Crit Care Med 1998, 157: 4-9
- 73. de Monchy JG, Kauffman HF, Venge P, Koeter GH, Jansen HM, Sluiter HJ, and de Vries K: Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. Am Rev Respir Dis 1985, 131: 373-376

- 74. O'Byrne PM, Dolovich J, and Hargreave FE: Late asthmatic responses. Am Rev Respir Dis 1987, 136: 740-751
- 75. Gauvreau GM, Lee JM, Watson RM, Irani AA, Schwartz LB, and O'Byrne PM: Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. Am J Respir Crit Care Med 2000, 161: 1473-1478
- 76. de Kluijver J, Evertse CE, Schrumpf JA, van der Veen H, Zwinderman AH, Hiemstra PS, Rabe KF, and Sterk PJ: Asymptomatic Worsening of Airway Inflammation during Low-Dose Allergen Exposure in Asthma: Protection by Inhaled Steroids. Am J Respir Crit Care Med 2002, 166: 294-300
- 77. Wood LJ, Inman MD, Watson RM, Foley R, Denburg JA, and O'Byrne PM: Changes in bone marrow inflammatory cell progenitors after inhaled allergen in asthmatic subjects. Am J Respir Crit Care Med 1998, 157: 99-105
- 78. Gauvreau GM, Watson RM, Rerecich TJ, Baswick E, Inman MD, and O'Byrne PM: Repeatability of allergen-induced airway inflammation. J Allergy Clin Immunol 1999, 104: 66-71
- 79. Gauvreau GM, Watson RM, and O'Byrne PM: Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. Am J Respir Crit Care Med 1999, 160: 640-647
- 80. Platts-Mills TA, Tovey ER, Mitchell EB, Moszoro H, Nock P, and Wilkins SR: Reduction of bronchial hyperreactivity during prolonged allergen avoidance. Lancet 1982, 2: 675-678
- van Rensen EL, Straathof KCM, Veselic-Charvat M, Zwinderman AH, Bel EH, and Sterk PJ: Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. Thorax 1999, 54: 403-408
- 82. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, and Holgate ST: Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. Am Rev Respir Dis 1992, 145: 669-674

- 83. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, and Johansson SA: Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. Am Rev Respir Dis 1992, 145: 890-899
- 84. van Grunsven PM, van Schayck CP, Molema J, Akkermans RP, and van Weel C: Effect of inhaled corticosteroids on bronchial responsiveness in patients with "corticosteroid naive" mild asthma: a meta-analysis. Thorax 1999, 54: 316-322
- 85. Gauvreau GM, Sulakvelidze I, Watson RM, Inman MD, Rerecich TJ, and O'Byrne PM: Effects of once daily dosing with inhaled budesonide on airway hyperresponsiveness and airway inflammation following repeated low-dose allergen challenge in atopic asthmatics. Clin Exp Allergy 2000, 30: 1235-1243
- 86. Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, and Chakir J: Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence Of inhaled corticosteroids. Am J Respir Crit Care Med 2000, 162: 1308-1313
- 87. Dahlen S-E, Hedqvist P, Hammarstrom S, and Samuelsson B: Leukotrienes are potent constrictors of human bronchi. Nature 1980, 286: 484-486
- 88. Lewis RA, Austen KF, and Soberman RJ: Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. N Engl J Med 1990, 323: 645-655
- 89. Drazen JM, Israel E, and O'Byrne PM: Treatment of Asthma with Drugs Modifying the Leukotriene Pathway. N Engl J Med 1999, 340: 197-206
- 90. Dworski R, Fitzgerald GA, Oates JA, and Sheller JR: Effect of oral prednisone on airway inflammatory mediators in atopic asthma. Am J Respir Crit Care Med 1994, 149: 953-959
- 91. Hamilton A, Faiferman I, Stober P, Watson RM, and O'Byrne PM: Pranlukast, a cysteinyl leukotriene receptor antagonist, attenuates allergen-induced early- and late-phase bronchoconstriction and airway hyperresponsiveness in asthmatic subjects. J Allergy Clin Immunol 1998, 102: 177-183

- 92. Gibson PG, Dolovich J, Denburg JA, Ramsdale EH, and Hargreave FE: Chronic cough: eosinophilic bronchitis without asthma. Lancet 1989, 1: 1346-1348
- 93. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, and Kay AB: Bronchial biopsies in asthma:An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989, 140: 1745-1753
- 94. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, and Barnes PJ: Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000, 356: 2144-2148
- 95. Flood-Page PT, Menzies-Gow AN, Kay AB, and Robinson DS: Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. Am J Respir Crit Care Med 2003, 167: 199-204
- 96. Boulet LP, Chakir J, Dube J, Laprise C, Boutet M, and Laviolette M: Airway inflammation and structural changes in airway hyper- responsiveness and asthma: an overview. Can Respir J 1998, 5: 16-21
- 97. Elias JA, Zhu Z, Chupp G, and Homer RJ: Airway remodeling in asthma. J Clin Invest 1999, 104: 1001-1006
- 98. Vignola AM, Chanez P, Bonsignore G, Godard P, and Bousquet J: Structural consequences of airway inflammation in asthma. J Allergy Clin Immunol 2000, 105: S514-S517
- 99. Homer RJ and Elias JA: Consequences of long-term inflammation. Airway remodeling. Clin Chest Med 2000, 21: 331-43, ix
- 100. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, and Zhu Z: New insights into the pathogenesis of asthma. J Clin Invest 2003, 111: 291-297
- 101. Wang L, McParland BE, and Pare PD: The functional consequences of structural changes in the airways: implications for airway hyperresponsiveness in asthma. Chest 2003, 123: 356S-362S

- 102. McParland BE, Macklem PT, and Pare PD: Airway wall remodeling: friend or foe? J Appl Physiol 2003, 95: 426-434
- Ellis A: The pathological anatomy of bronchial asthma. Am J Med Sci 1908, 136: 407-429
- 104. Huber H and Koessler K: The pathology of bronchial asthma. Arch Intern Med 1922, 30: 689-760
- 105. Reid LM: The embryology of the lung. Ciba Foundation Symposium of Development of the Lung. Edited by Reuck AVS and Porter R. London, J. & A. Churchill, 1966, pp. 109-124
- 106. Reid LM: The pulmonary circulation: remodeling in growth and disease. The 1978 J. Burns Amberson lecture. Am Rev Respir Dis 1979, 119: 531-546
- 107. Bousquet J, Chanez P, Lacoste JY, White R, Vic P, Godard P, and Michel FB: Asthma: a disease remodeling the airways. Allergy 1992, 47: 3-11
- 108. Elias JA, Zhu Z, Chupp G, and Homer RJ: Airway remodeling in asthma. J Clin Invest 1999, 104: 1001-1006
- 109. Davies DE, Wicks J, Powell RM, Puddicombe SM, and Holgate ST: Airway remodeling in asthma: new insights. J Allergy Clin Immunol 2003, 111: 215-225
- 110. Dunnil MS: The pathology of asthma with special references to changes in the bronchial mucosa. J Clin Pathol 1960, 13: 27-32
- 111. Dunnill MS, Massarella GR, and Anderson JA: A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. Thorax 1969, 24: 176-179
- 112. Takizawa T and Thurlbeck WM: Muscle and mucous gland size in the major bronchi of patients with chronic bronchitis, asthma, and asthmatic bronchitis. Am Rev Respir Dis 1971, 104: 331-336

- 113. Hossain S: Quantitative measurement of bronchial muscle in men with asthma. Am Rev Respir Dis 1973, 107: 99-109
- 114. Sobonya RE: Quantitative structural alterations in long-standing allergic asthma. Am Rev Respir Dis 1984, 130: 289-292
- 115. James AL, Pare PD, and Hogg JC: The mechanics of airway narrowing in asthma. Am Rev Respir Dis 1989, 139: 242-246
- 116. Saetta M, di Stefano A, Rosina C, Thiene G, and Fabbri LM: Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. Am Rev Respir Dis 1991, 143: 138-143
- 117. Carroll N, Elliot J, Morton A, and James A: The structure of large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis 1993, 147: 405-410
- 118. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, and Kay AB: Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989, 140: 1745-1753
- 119. Djukanovic R, Roche WR, Wilson JW, Beasley CRW, Twentyman OP, Howarth PH, and Holgate ST: Mucosal inflammation in asthma. Am Rev Respir Dis 1990, 142: 434-457
- 120. Chetta A, Foresi A, Del Donno M, Bertorelli G, Pesci A, and Olivieri D: Airways remodeling is a distinctive feature of asthma and is related to severity of disease. Chest 1997, 111: 852-857
- 121. Jeffery PK, Laitinen A, and Venge P: Biopsy markers of airway inflammation and remodelling. Respir Med 2000, 94 Suppl F: S9-15
- 122. Jeffery PK: Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001, 164: S28-S38
- 123. Hogg JC: The pathology of asthma. Clin Chest Med 1984, 5: 567-571

- 124. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, and Kay AB: Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989, 140: 1745-1753
- 125. Roche WR, Beasley R, Williams JH, and Holgate ST: Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1989, 1: 520-524
- 126. Djukanovic R, Lai CK, Wilson JW, Britten KM, Wilson SJ, Roche WR, Howarth PH, and Holgate ST: Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. Eur Respir J 1992, 5: 538-544
- 127. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, and Johansson SA: Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. Am Rev Respir Dis 1992, 145: 890-899
- 128. Ebina M, Takahashi T, Chiba T, and Motomiya M: Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. Am Rev Respir Dis 1993, 148: 720-726
- 129. Huang J, Olivenstein R, Taha R, Hamid Q, and Ludwig M: Enhanced Proteoglycan Deposition in the Airway Wall of Atopic Asthmatics. Am J Respir Crit Care Med 1999, 160: 725-729
- 130. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, and Fahy JV: Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. Am J Respir Crit Care Med 2001, 163: 517-523
- 131. James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, and Carroll NG: The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. Am J Respir Crit Care Med 2002, 166: 1590-1595
- 132. Chetta A, Foresi A, Del Donno M, Consigli GF, Bertorelli G, Pesci A, Barbee RA, and Olivieri D: Bronchial responsiveness to distilled water and methacholine and its relationship to inflammation and remodeling of the airways in asthma. Am J Respir Crit Care Med 1996, 153: 910-917

- 133. Richter A, Puddicombe SM, Lordan JL, Bucchieri F, Wilson SJ, Djukanovic R, Dent G, Holgate ST, and Davies DE: The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. Am J Respir Cell Mol Biol 2001, 25: 385-391
- 134. Postlethwaite AE, Holness MA, Katai H, and Raghow R: Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. J Clin Invest 1992, 90: 1479-1485
- 135. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Canonica GW, Jasmin C, and Azzarone B: Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. J Clin Invest 1998, 101: 2129-2139
- 136. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, and Elias JA: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999, 103: 779-788
- 137. Aubert J-D, Dalal BI, Bai TR, Roberts CR, Hayashi S, and Hogg JC: Transforming growth factor $\beta 1$ gene expression in human airways. Thorax 1994, 49: 225-232
- 138. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, O'Byrne P, Tamura G, Jordana M, and Shirato K: Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. Am J Respir Cell Mol Biol 1996, 15: 404-409
- 139. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, and Hamid Q: Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. Am J Respir Cell Mol Biol 1997, 17: 326-333
- 140. Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, and Bousquet J: Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med 1997, 156: 591-599

- 141. Aubert JD, Hayashi S, Hards J, Bai TR, Pare PD, and Hogg JC: Platelet-derived growth factor and its receptor in lungs from patients with asthma and chronic airflow obstruction. Am J Physiol 1994, 266: L655-L663
- 142. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, O'Byrne P, Dolovich J, Jordana M, Tamura G, and .: Eosinophils as a potential source of platelet-derived growth factor B-chain (PDGF-B) in nasal polyposis and bronchial asthma. Am J Respir Cell Mol Biol 1995, 13: 639-647
- 143. Hagaman DD, Okayama Y, D'Ambrosio C, Prussin C, Gilfillan AM, and Metcalfe DD: Secretion of interleukin-1 receptor antagonist from human mast cells after immunoglobulin E-mediated activation and after segmental antigen challenge. Am J Respir Cell Mol Biol 2001, 25: 685-691
- 144. Bosse M, Chakir J, Rouabhia M, Boulet LP, Audette M, and Laviolette M: Serum Matrix Metalloproteinase-9:Tissue Inhibitor of Metalloproteinase-1 Ratio Correlates with Steroid Responsiveness in Moderate to Severe Asthma. Am J Respir Crit Care Med 1999, 159: 596-602
- 145. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, D'accardi P, Bousquet J, and Bonsignore G: Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. Am J Respir Crit Care Med 1999, 158: 1945-1950
- 146. Hirst SJ, Barnes PJ, and Twort CH: Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. Am J Respir Cell Mol Biol 1992, 7: 574-581
- 147. Desmouliere A, Geinoz A, Gabbiani F, and Gabbiani G: Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 1993, 122: 103-111
- 148. Sime PJ, Xing Z, Graham FL, Csaky KG, and Gauldie J: Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest 1997, 100: 768-776

- 149. Zhu Z, Lee CG, Zheng T, Chupp G, Wang J, Homer RJ, Noble PW, Hamid Q, and Elias JA: Airway inflammation and remodeling in asthma. Lessons from interleukin 11 and interleukin 13 transgenic mice. Am J Respir Crit Care Med 2001, 164: S67-S70
- 150. Salmon M, Walsh DA, Koto H, Barnes PJ, and Chung KF: Repeated allergen exposure of sensitized Brown-Norway rats induces airway cell DNA synthesis and remodelling. Eur Respir J 1999, 14: 633-641
- 151. Palmans E, Kips JC, and Pauwels RA: Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 2000, 161: 627-635
- 152. Wang ZL, Walker BA, Weir TD, Yarema MC, Roberts CR, Okazawa M, Pare PD, and Bai TR: Effect of chronic antigen and beta 2 agonist exposure on airway remodeling in guinea pigs. Am J Respir Crit Care Med 1995, 152: 2097-2104
- 153. Foster PS, Ming Y, Matthei KI, Young IG, Temelkovski J, and Kumar RK: Dissociation of inflammatory and epithelial responses in a murine model of chronic asthma. Lab Invest 2000, 80: 655-662
- 154. Henderson WR, Jr., Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, and Chi EY: A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. Am J Respir Crit Care Med 2002, 165: 108-116
- 155. Sapienza S, Du T, Eidelman DH, Wang NS, and Martin JG: Structural changes in the airways of sensitized brown Norway rats after antigen challenge. Am Rev Respir Dis 1991, 144: 423-427
- 156. Moreno RH, Hogg JC, and Pare PD: Mechanics of airway narrowing. Am Rev Respir Dis 1986, 133: 1171-1180
- 157. Wiggs BR, Bosken C, Pare PD, James A, and Hogg JC: A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1992, 145: 1251-1258

- 158. Lambert RK, Wiggs BR, Kuwano K, Hogg JC, and Pare PD: Functional significance of increased airway smooth muscle in asthma and COPD. J Appl Physiol 1993, 74: 2771-2781
- 159. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, and Hogg JC: Small airways dimensions in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1993, 148: 1220-1225
- 160. Macklem PT: A theoretical analysis of the effect of airway smooth muscle load on airway narrowing. Am J Respir Crit Care Med 1996, 153: 83-89
- 161. Wiggs BR, Moreno R, Hogg JC, Hilliam C, and Pare PD: A model of the mechanics of airway narrowing. J Appl Physiol 1990, 69: 849-860
- 162. Lambert RK, Codd SL, Alley MR, and Pack RJ: Physical determinants of bronchial mucosal folding. J Appl Physiol 1994, 77: 1206-1216
- 163. Seow CY, Wang L, and Pare PD: Airway narrowing and internal structural constraints. J Appl Physiol 2000, 88: 527-533
- 164. Wiggs BR, Hrousis CA, Drazen JM, and Kamm RD: On the mechanism of mucosal folding in normal and asthmatic airways. J Appl Physiol 1997, 83: 1814-1821
- 165. Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, and Boutet M: Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. Chest 1997, 112: 45-52
- 166. Laprise C, Laviolette M, Boutet M, and Boulet LP: Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling. Eur Respir J 1999, 14: 63-73
- 167. Pohunek, P., Roche, W. R., Tarzikova, J., Kurdmann, J., and Warner, J. O. Eosinophilic inflammation in the bronchial mucosa in children with bronchial asthma [abstract]. Eur Respir J 11[suppl 25]. 2000.

- 168. Wanner A, Abraham WM, Douglas JS, Drazen JM, Richerson HB, and Ram JS: NHLBI Workshop Summary. Models of airway hyperresponsiveness. Am Rev Respir Dis 1990, 141: 253-257
- 169. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, and Gelfand EW: Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med 1997, 156: 766-775
- 170. Renz H, Smith HR, Henson JE, Ray BS, Irvin CG, and Gelfand EW: Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. J Allergy Clin Immunol 1992, 89: 1127-1138
- 171. Gavett SH, Chen X, Finkelman F, and Wills-Karp M: Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. Am J Respir Cell Mol Biol 1994, 10: 587-593
- 172. Kips JC, Brusselle GJ, Joos GF, Peleman RA, Tavernier JH, Devos RR, and Pauwels RA: Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice. Am J Respir Crit Care Med 1996, 153: 535-539
- 173. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, and Locksley RM: Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J Exp Med 1996, 183: 109-117
- 174. Irvin CG, Tu YP, Sheller JR, and Funk CD: 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. Am J Physiol 1997, 272: L1053-L1058
- 175. Hogan SP, Mould A, Kikutani H, Ramsay AJ, and Foster PS: Aeroallergeninduced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. J Clin Invest 1997, 99: 1329-1339

- 176. Hamelmann E, Oshiba A, Schwarze J, Bradley K, Loader J, Larsen GL, and Gelfand EW: Allergen-specific IgE and IL-5 are essential for the development of airway hyperresponsiveness. Am J Respir Cell Mol Biol 1997, 16: 674-682
- 177. Erb KJ, Holloway JW, Sobeck A, Moll H, and Le Gros G: Infection of mice with Mycobacterium bovis-Bacillus Calmette-Guerin (BCG) suppresses allergeninduced airway eosinophilia. J Exp Med 1998, 187: 561-569
- 178. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, and Donaldson DD: Interleukin-13: central mediator of allergic asthma. Science 1998, 282: 2258-2261
- 179. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, and Corry DB: Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998, 282: 2261-2263
- 180. Hogan SP, Matthaei KI, Young JM, Koskinen A, Young IG, and Foster PS: A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. J Immunol 1998, 161: 1501-1509
- 181. Coyle AJ, Kohler G, Tsuyuki S, Brombacher F, and Kopf M: Eosinophils are not required to induce airway hyperresponsiveness after nematode infection. Eur J Immunol 1998, 28: 2640-2647
- 182. Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, and Locksley RM: Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. Molecular Medicine 1998, 4: 344-355
- 183. Wills-Karp M: Immunologic basis of antigen-induced airway hyperresponsiveness. Annual Review of Immunology 1999, 17: 255-281
- 184. Hamelmann E, Takeda K, Haczku A, Cieslewicz G, Shultz L, Hamid Q, Xing Z, Gauldie J, and Gelfand EW: Interleukin (IL)-5 but not immunoglobulin E reconstitutes airway inflammation and airway hyperresponsiveness in IL-4deficient mice. Am J Respir Cell Mol Biol 2000, 23: 327-334

- 185. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, and Foster PS: Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. J Immunol 2000, 165: 108-113
- 186. Mattes J, Yang M, Siqueira A, Clark K, MacKenzie J, McKenzie AN, Webb DC, Matthaei KI, and Foster PS: IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. J Immunol 2001, 167: 1683-1692
- 187. Inman MD, Ellis R, Wattie J, Denburg JA, and O'Byrne PM: Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. Am J Respir Cell Mol Biol 1999, 21: 473-479
- 188. Hessel EM, Van Oosterhout AJ, Van A, I, Van Esch B, Hofman G, Van Loveren H, Savelkoul HF, and Nijkamp FP: Development of airway hyperresponsiveness is dependent on interferon- gamma and independent of eosinophil infiltration. Am J Respir Cell Mol Biol 1997, 16: 325-334
- 189. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, and Young IG: Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 1996, 183: 195-201
- 190. Wang J, Palmer K, Lotvall J, Milan S, Lei XF, Matthaei KI, Gauldie J, Inman MD, Jordana M, and Xing Z: Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia. J Clin Invest 1998, 102: 1132-1141
- 191. Tanaka H, Nagai H, and Maeda Y: Effect of anti-IL-4 and anti-IL-5 antibodies on allergic airway hyperresponsiveness in mice. Life Sci 1998, 62: L169-L174
- 192. Gavett SH, O'Hearn DJ, Karp CL, Patel EA, Schofield BH, Finkelman FD, and Wills-Karp M: Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. Am J Physiol 1997, 272: L253-L261
- 193. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, and Kumar RK: An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax 1998, 53: 849-856

- 194. Trifilieff A, El Hashim A, and Bertrand C: Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. Am J Physiol Lung Cell Mol Physiol 2000, 279: L1120-L1128
- 195. Blyth DI, Wharton TF, Pedrick MS, Savage TJ, and Sanjar S: Airway subepithelial fibrosis in a murine model of atopic asthma: suppression by dexamethasone or anti-interleukin-5 antibody. Am J Respir Cell Mol Biol 2000, 23: 241-246
- 196. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL, and Lukacs NW: Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. Am J Pathol 2000, 156: 723-732
- 197. Tanaka H, Masuda T, Tokuoka S, Takahashi Y, Komai M, Nagao K, and Nagai H: Time course study on the development of allergen-induced airway remodeling in mice: the effect of allergen avoidance on established airway remodeling. Inflamm Res 2002, 51: 307-316
- 198. Kenyon NJ, Ward RW, and Last JA: Airway fibrosis in a mouse model of airway inflammation. Toxicol Appl Pharmacol 2003, 186: 90-100
- 199. Masuda T, Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwara D, and Nagai H: Mast cells play a partial role in allergen-induced subepithelial fibrosis in a murine model of allergic asthma. Clin Exp Allergy 2003, 33: 705-713
- 200. Moir LM, Leung SY, Eynott PR, McVicker CG, Ward JP, Chung KF, and Hirst SJ: Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. Am J Physiol Lung Cell Mol Physiol 2003, 284: L148-L159

Figure Legends

Figure 1

Response of FEV_1 to increasing concentrations of either methacholine or histamine in normal subjects and in asthmatic patients with increasing severity of AHR. PC₂₀ refers to provocative concentration of methacholine or histamine to cause a 20% fall in FEV₁

Figure 2

A schematic illustration of the potential mechanisms underlying both the *variable* and *sustained* components of airway hyperresponsiveness. These processes are thought to account for fluctuations in airway within asthmatic individuals and also for differences between patients with asthma and the normal population.

Figure 1



Inhaled Methacholine or Histamine (mg/ml)

Figure 2



Doubling Dose differences in MCh PC 20 (mg/ml)

CHAPTER 2

EFFFECTS OF MONTELUKAST AND BUDESONIDE ON AIRWAY RESPONSES AND AIRWAY INFLAMMATION IN ASTHMA

Leigh R, Vethanayagam D, Yoshida M, Watson RM, Rerecich T, Inman MD, O'Byrne PM.

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Richard Leigh's contribution:

I am first author on this manuscript, and I was responsible for the recruitment of study participants, the collection, management and analysis of clinical data, and for the writing and subsequent revisions of the final manuscript. I was involved in all of the clinical study procedures, and performed methacholine and allergen inhalation challenge tests and sputum induction procedures on the majority of study subjects. I am completely familiar with all aspects of data collection, and all those who assisted me did so under my direction. The writing of the final manuscript was solely my responsibility, and the figures and tables in this paper are my own work. Dilini Vethanayagam and Makoto Yoshida were involved in the study design, and provided feedback during the preparation of the manuscript. Rick Watson assisted in all the clinical study procedures, and Tracy Rerecich was responsible for processing and performing all sputum cell counts. Mark Inman was involved in the study design, provided valuable statistical advice, and provided regular feedback during the preparation of the manuscript. Paul O'Byrne provided the academic environment, laboratory facilities, and financial support to carry out the study. As senior author, he was also integral to the study design, the supervision of data collection and analysis, and to the supervision of the writing and subsequent revisions of the final manuscript.



Firestone Institute for Respiratory Health



April 20, 2004

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Dear Ms. Sheppard

I am completing a PhD thesis at McMaster University entitled, "The Uncoupling of the inflammatory and Structural Components of Airway Hyperresponsiveness", and I would like your permission to reprint the following journal article in my thesis:

Leigh R, Vethanayagam D, Yoshida M, Watson RM, Rerecich T, Inman MD, O'Byrne PM. Effects of montelukasi and budesonide on airway responses and airway inflammation in asthma. Am J Respir Crit Care Med 2002; 166: 1212-7. A photocopy of the article is enclosed for your reference.

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Effects of Montelukast and Budesonide on Airway Responses and Airway Inflammation in Asthma

Richard Leigh, Dilini Vethanayagam, Makoto Yoshida, Richard M. Watson, Tracy Rerecich, Mark D. Inman, and Paul M. O'Byrne

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Inhaled corticosteroids are effective antiinflammatory therapy for asthma; however, they do not completely abolish allergen-induced airway inflammation. Leukotriene modifiers attenuate both early and late allergen responses and have antiinflammatory properties. We reasoned that treatment with budesonide and montelukast in combination might provide greater antiinflammatory effects than either drug alone, and the purpose of this study was to compare the effects of treatment with budesonide and montelukast, alone or in combination, on outcome variables after allergen inhalation. Ten subjects with asthma with dual responses after allergen inhalation were included in this randomized, double-blind, crossover study. Outcomes included early and late asthmatic responses, and changes in airway responsiveness and sputum eosinophilia, measured before and after challenge. Treatment with montelukast attenuated the maximal early asthmatic response compared with placebo (p < 0.001) and budesonide (p = 0.002). Both budesonide and montelukast, alone and in combination, attenuated the maximal late asthmatic response compared with placebo (p < 0.01). Budesonide and montelukast, alone and in combination, afforded protection against allergen-induced airway hyperresponsiveness (p < 0.05), although the treatment effect of budesonide was greater than that of montelukast (p < 0.05). Treatment with budesonide and montelukast, alone and in combination, also attenuated allergen-induced sputum eosinophilia. Thus, montelukast and budesonide attenuated allergen-induced asthmatic responses, airway hyperresponsiveness, and sputum eosinophilia, although combination treatment did not provide greater antiinflammatory effects than either drug alone.

Keywords: asthma; allergen challenge; airway inflammation; glucocorticosteroids; cysteinyl leukotrienes.

Inhaled corticosteroids (ICSs) are regarded as the cornerstone of effective antiinflammatory therapy for the treatment of asthma (1). Regular long-term ICS therapy significantly reduces asthma mortality (2), asthma exacerbations (3, 4), airway inflammation, and asthma symptoms (5, 6) and significantly improves lung function in subjects with asthma (7).

Although regular ICS treatment inhibits allergen-induced late airway responses and attenuates allergen-induced airway hyperresponsiveness (AHR), it does not completely abolish allergen-induced increases in sputum eosinophils (8, 9). Furthermore, a recent study from the Asthma Clinical Research

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Network (10) has demonstrated that there is significant intersubject variability in response to low-medium dose ICS treatment, suggesting that at least in subjects demonstrating a poor response to ICS, there are ongoing inflammatory mechanisms that are relatively insensitive to the antiinflammatory effects of ICS.

Among the various proinflammatory mediators involved in the pathophysiology of asthma, cysteinyl leukotrienes have a causative role in mediating bronchoconstriction and allergic airway inflammation (11–13). Cysteinyl leukotrienes are released from inflammatory cells in the airways and induce bronchoconstriction (14), inflammatory cell infiltration (15– 16), smooth muscle proliferation (17), mucous secretion, and increased vascular permeability (12, 18, 19). However, *in vivo* studies have shown that the synthesis and release of cysteinyl leukotrienes into the airways of patients with asthma are not blocked by corticosteroid therapy (20), suggesting that the inflammatory effects of cysteinyl leukotrienes are not inhibited by corticosteroid treatment.

Montelukast sodium (Singulair) is a potent, oral, specific cysteinyl leukotriene D_4 -receptor antagonist that inhibits bronchoconstrictor response to exercise (21) and early and late airway responses to allergen (22). Furthermore, montelukast has been shown to attenuate airway eosinophilia in subjects with asthma having eosinophilic bronchitis (23), whereas the related leukotriene-modifying compounds, zafirlukast and zileuton, reduce airway eosinophilia in bronchoalveolar lavage fluid after segmental allergen challenge (24, 25).

We reasoned that the combination of a low dose of ICS and a cysteinyl leukotriene receptor antagonist (montelukast) may provide greater antiinflammatory effects against allergen-induced airway inflammation than either drug alone. Therefore, the primary purpose of this study was to evaluate the effects of budesonide and montelukast, alone and in combination, on allergen-induced early and late bronchoconstrictor responses, AHR, and airway inflammation in subjects with mild, stable asthma.

METHODS

Subjects

Ten nonsmoking subjects (seven males, three females) (Table 1) with stable, mild atopic asthma were included in the study. All subjects had symptoms of asthma for more than a year, and their inclusion in the study was based on their having a provocative concentration producing a 20% reduction in FEV₁ (PC₂₀) of less than 16 mg/ml methacholine (MCh) and allergen-induced early and late bronchoconstrictor responses of at least 15% reduction in FEV₁ during screening challenges. Subjects who had been treated with any asthma medication other than inhaled β_2 -agonists, or who used inhaled β_2 -agonists more frequently than once daily during the 4-week period before screening, were not permitted to participate in the study. One subject failed to complete all treatment arms of the study due to a protocol violation, and the analysis was thus based on data from nine subjects. The study protocol was approved by the Ethics Committee at the McMaster University

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TABLE 1. SUBJECT CHARACTERISTICS

	Mean (Range)
Age, yr	37 (20-52)
Weight, kg	77.8 (58–100)
Height, cm	172 (160–181)
FEV ₁ , L	3.1 (2.0-3.8)
FEV ₁ , % predicted	82 (72–96)
FEV1/VC, %	75 (68–90)
PC ₂₀ methacholine, mg/ml*	2.9 (0.15–9.98)

Definition of abbreviations: PC_{20} = provocative concentration producing a 20% reduction in FEV₁; VC = vital capacity.

* Geometric mean.

Health Sciences Centre, and all subjects gave written, informed consent to participate in the study.

Study Design and Protocol

The study was performed using a prospective, double-blind, doubledummy, placebo-controlled, randomized, four-period crossover design. After successful screening, subjects were randomized to receive one of four different treatment regimens of 10 days each, separated by washout periods of at least 21 days (Figure 1). The four treatment regimens were (1) montelukast (Singulair) 10 mg, received orally once daily in the evening and placebo Turbuhaler; (2) inhaled budesonide (Pulmicort Turbuhaler) 200 μ g, one inhalation taken in the morning and evening (total daily dose, 400 μ g) and placebo tablet; (3) active montelukast and active budesonide in dosages described previously; and (4) placebo tablet and placebo Turbuhaler.

On the first day of each treatment period, an MCh inhalation challenge and sputum induction were performed to determine baseline pretreatment airway responsiveness and airway inflammatory status, respectively (Day 1). Subjects began receiving their study medications on the evening of Day 1, continued thereafter, and on Day 8, the MCh challenge and sputum induction were repeated to establish preallergen baseline levels. On Day 9, after receiving the morning dose of study medication, subjects underwent an allergen inhalation challenge; spirometry was monitored throughout the day to check for early and late asthmatic responses. Seven hours after allergen inhalation, sputum induction was repeated to assess airway inflammation. The final dose of study medication was received on the morning of Day 10, after which MCh challenge and sputum induction were performed, 24 hours after allergen inhalation. All measurements for each subject were made at the same time of the day (± 2 hours) at baseline and after each treatment period. Study subjects were allowed to use inhaled B2-agonists on an as-needed basis throughout the treatment and washout periods but were asked to refrain from doing so 8 hours before any study visit.

Randomization and Allocation Concealment

Randomization was performed by using computer-generated randomization codes, which were maintained by a research pharmacist at McMaster University who was independent of the study. Treatment allocation was concealed from the investigators and participants for



Figure 1. The protocol used within each treatment period of the study. The four treatment periods were each separated by a washout period of at least 21 days.

the duration of the study. All study medications were independently packaged and labeled by the hospital pharmacy. Placebo tablets were identical in appearance and labeling to montelukast tablets, both of which were supplied by Merck Research Laboratories (Merck Frosst Canada & Co., Montreal, PQ, Canada). Placebo Turbuhalers were identical in appearance and labeling to budesonide Turbuhalers (Pulmicort Turbuhaler, 200 μ g/dose), both of which were supplied by Astra-Zeneca (AstraZeneca Canada Inc., Mississauga, ON, Canada). At the start of a treatment period, each subject was given a new coded container with sufficient study tablets and a new coded Turbuhaler. Inhaler technique was checked at each visit and corrected if necessary. At the end of each treatment period, study medication was returned, and compliance was monitored by counting the number of tablets and Turbuhaler doses remaining.

Outcome Measurements

The primary outcome was the effect of treatment on allergen-induced airway eosinophilia. Secondary outcomes were the effects of treatment on allergen-induced early and late bronchoconstrictor responses and on allergen-induced AHR. The sample size was considered sufficient because previous studies have shown that a group of eight or more subjects has sufficient power to demonstrate differences in allergeninduced airway eosinophilia and in allergen-induced early and late asthmatic responses, using the same methodologies used in this study (8, 26).

Laboratory Procedures

MCh inhalation challenge. MCh inhalation challenge was performed using the method described by Cockcroft and colleagues (27). Subjects inhaled through a mouthpiece attached to a Wright nebulizer (Roxon Medi-Tech, Montreal, PQ, Canada). Normal saline, followed by doubling concentration increases in MCh were nebulized for 2 minutes each. FEV₁ was measured at 30, 90, 180, and 300 seconds after each inhalation using a Collins water-sealed spirometer (Warren E. Collins, Braintree, MA) and kymograph. The test was terminated when FEV₁ had fallen to a level at least 20% below the postsaline measurement. The concentration of MCh required to achieve a decrease in FEV₁ of 20% (MCh PC₂₀) was calculated through linear interpolation of percent fall in FEV₁ against the log-transformed MCh concentration (27).

Allergen inhalation challenge. Allergen challenge was performed according to the method described by O'Byrne and colleagues (28). The allergen producing the largest skin wheal diameter after skin prick testing was used for subsequent airway challenges. Allergens used were house dust mite (n = 5), cat (n = 4), and tree mix (combination of tree allergens indigenous to North America; viz., Ash, Birch, Hazelnut, Poplar, Oak mix, Willow, Maple, American Beech, Sycamore, and Elm) (n = 1). The concentration of allergen extract for inhalation was determined using a formula derived by Cockcroft and colleagues (29) using the results from skin test titrations and the MCh PC₂₀. During the screening allergen challenge, the starting concentration of allergen extract for inhalation was two doubling concentrations below that predicted to cause a 20% decrease in FEV₁. Doubling increases in allergen concentration were inhaled every 10 minutes until a 15% reduction in FEV₁ was achieved. FEV₁ was then measured at 10, 20, 30, 45, 60, 90, and 120 minutes after allergen inhalation, then each hour until 7 hours after allergen inhalation. The early bronchoconstrictor response was taken to be the largest percent fall in FEV1 within 2 hours after allergen inhalation, and the late bronchoconstrictor response was taken to be the largest percent fall in FEV₁ in the period beginning 3 hours and ending 7 hours after allergen inhalation. Maximal decreases in FEV1 were chosen to quantify the early and late response magnitudes based on earlier studies from our group, indicating that these measurements have utility in detecting treatment effects (30). Only subjects who achieved a 15% or greater early and late decline in FEV₁ on the allergen screening challenge were randomized, and the same allergen concentrations were used on all subsequent allergen inhalation challenges.

Sputum induction and analysis. Sputum induction was performed after MCh challenge on Days 1, 8, and 10, and 7 hours after allergen challenge on Day 9 of each treatment period. MCh challenge performed before sputum induction does not significantly alter the cellular and biochemical constituents of sputum (31). Sputum was induced as described by Pin and colleagues (32) and modified as described by Pizzichini and colleagues (33). Briefly, after pretreatment with inhaled albuterol (200 µg), subjects inhaled an aerosol of 3, 4, and 5% hypertonic saline for 7 minutes each from a Medix ultrasonic nebulizer (Clement Clarke, Harlow, Essex, UK). After each inhalation period, subjects expectorated sputum into a container. Sputum was processed within 2 hours of collection as described by Pizzichini and colleagues (34). Total cell counts were performed using a hemocytometer and were expressed as the number of cells per milliliter of sputum. Cells were resuspended in Dulbecco's phosphate buffered saline at 0.75 to 1.0×10^{6} /ml, and cytospins were prepared using a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickly, PA). Slides were stained using Diff-Quik (American Scientific Products, Mcgaw Park, IL), and a 400 nonsquamous differential cell count was performed on all slides by a single observer blind to the clinical data; the mean count from two slides per subject was used for analysis.

Statistical Analysis

Statistica software, version 5 (StatSoft, Inc., Tulsa, OK) was used to analyze the data. Measurement variability was expressed using the SD for baseline subject characteristics and SEM for outcome variables. MCh PC₂₀ measurements were log₂-transformed to normalize the data and are reported as geometric means (35). The choice of base 2 for the logarithmic transformation allows differences between PC₂₀ values to be expressed as doubling concentrations. Comparisons between treatment periods with respect to early and late asthmatic responses, AHR, and sputum eosinophils were made using two-factor repeatedmeasures analysis of variance to analyze the effect of the two independent variables, treatment and time, on the outcome variables described previously. Comparisons of treatment effects of placebo and the three active treatment regimens on allergen-induced airway responses were made using separate analyses of variance to compare the largest percent fall in FEV_1 during the early and late bronchoconstrictor responses. Similar comparisons were also made by analyzing area under the curve (26). Comparisons between treatment effects of placebo and the three treatment regimens on allergen-induced changes in MCh PC20 and sputum eosinophilia were made using separate analyses of variance. In the case of MCh $PC_{20},$ the pre- to postallergen delta ($\Delta)$ PC_{20} was calculated using the log₂-transformed data and is therefore expressed in the text as doubling concentration changes. Appropriate post hoc testing was performed using Duncan's test to assess for significant effects while controlling for multiple comparisons. All comparisons were two-tailed, and p values less than 0.05 were considered significant.

RESULTS

Early and Late Responses

There were no significant differences between the preallergen challenge FEV₁ values in each treatment arm, being 3.0 ± 0.38 L after placebo, 3.2 \pm 0.38 L after budesonide, 3.1 \pm 0.45 L after montelukast, and 3.1 ± 0.41 L after combination treatment with both drugs. The maximal early percent fall in FEV₁ after placebo treatment was $28.4 \pm 4.3\%$ (mean \pm SEM), which was not significantly attenuated by treatment with budesonide (25.3 \pm 7.0%), although it was significantly reduced by montelukast treatment alone to $12.4 \pm 3.9\%$ (p < 0.01) and by combination treatment with budesonide and montelukast to $11.0 \pm 3.7\%$ (p < 0.01). Whereas both montelukast treatment alone (p = 0.02)and the combination of montelukast and budesonide (p = 0.01)resulted in a significant attenuation of the maximal early percent fall in FEV_1 when compared with budesonide treatment alone. there were no significant differences between treatment with montelukast alone and in combination with budesonide on the maximal early percent fall in FEV_1 (Figure 2).

The maximal late percent fall in FEV₁ after placebo treatment was 17.8 \pm 4.6%. This was significantly reduced by all three treatments, to 3.4 \pm 3.0% after budesonide (p < 0.001), 7.8 \pm 3.3% after montelukast (p = 0.01), and 3.0 \pm 2.6% after the combination of montelukast and budesonide (p < 0.001). There were no significant differences in the degree of attenuation be-



Figure 2. Time course of the mean decline in FEV₁ (expressed as percent below the prechallenge values), after allergen challenge in each treatment period. There was significant attenuation of the maximal early response after treatment with either montelukast or the combination of budesonide and montelukast when compared with placebo (p < 0.01). There was also significant attenuation of the maximal late response by all three active treatment regimens when compared with placebo (p < 0.01).

tween budesonide and montelukast, either alone or in combination, on the maximal late percent fall in FEV_1 (Figure 2). Similar significant differences between treatment groups were observed for both the early and late responses when the data were analyzed using the area under the curve.

Airway Responsiveness

After treatment, but before allergen inhalation (Day 8), the MCh PC₂₀ increased relative to pretreatment values by 0.82 \pm 0.45 doubling concentrations after treatment with budesonide, by 0.52 ± 0.29 doubling concentrations after treatment with montelukast, and by 0.32 ± 0.29 doubling concentrations after treatment with the combination of budesonide and montelukast. However, these increases were not significantly different from the 0.27 \pm 0.25 doubling concentration increase after placebo treatment (p > 0.05) (Figure 3). The allergen-induced decreases in MCh PC₂₀ after treatment with budesonide, montelukast, and the combination of budesonide and montelukast were 0.46 \pm 0.39, 0.81 \pm 0.28, and 0.47 \pm 0.33 doubling concentrations, respectively, which were all significantly less than the 1.49 ± 0.38 doubling concentration decrease after allergen challenge in the placebo treatment phase (p < 0.05) (Figure 3). Furthermore, the allergen-induced decrease in MCh PC₂₀ after treatment with budesonide alone (0.46 ± 0.39) was significantly less than the allergen-induced decrease observed after treatment with montelukast alone (0.81 ± 0.28) (p < 0.05).

Sputum Eosinophilia

The percentage of sputum cosinophils was not significantly different at the pretreatment visit or at the preallergen visit, being $1.95 \pm 0.94\%$ after treatment with placebo, $0.86 \pm 0.28\%$ with budesonide, $0.96 \pm 0.47\%$ with montelukast, and $0.74 \pm 0.88\%$ after combination treatment with both drugs (p > 0.05) (Figure 4). A significant increase in sputum cosinophilia was measured 7 hours after allergen inhalation ($8.10 \pm 3.01\%$) after treatment with placebo (p < 0.01). This allergen-induced sputum cosinophilia was attenuated by treatment with budesonide



Figure 3. Airway responsiveness, as measured by MCh PC₂₀, before and after treatment and allergen challenge in each treatment period of the study. *Indicates a significant difference from pre-allergen value in the same treatment group (p < 0.001). #Indicates a significant difference from placebo at the same time point (p < 0.05). +Indicates that bude-sonide was significantly different from montelukast at the same time point (p < 0.05).

 $(2.95 \pm 1.24\%)$, montelukast $(3.80 \pm 1.35\%)$, and by treatment with montelukast and budesonide in combination $(4.17 \pm 1.55\%)$, all of which were significantly different when compared with placebo (p < 0.05) (Figure 4). Similarly, a significant increase in sputum eosinophilia was measured 24 hours after allergen inhalation after placebo treatment $(12.94 \pm 4.72\%)$ (p < 0.001), and this was again attenuated by treatment with budesonide (4.85 ± 1.60%), montelukast (3.21 ± 1.62%), and by the combination of montelukast and budesonide (3.72 ± 1.05%), all of which were significantly different from placebo (p < 0.001) (Figure 4). There were no significant differences in the ability of budesonide or montelukast, either alone or in combination, to attenuate sputum eosinophilia at either 7 hours or 24 hours after allergen challenge (p > 0.05). Similar differences were



Figure 4. The percentage of sputum eosinophils before and after treatment and allergen challenge in each treatment period of the study. *Indicates significant difference from preallergen value in the same treatment group (p < 0.01). *Filled triangles* indicate a significant difference from placebo at the same time point (p < 0.05).

ate different components of the inflammatory pathway, then the combination of budesonide and montelukast might have been expected to show enhanced attenuation of allergen-induced hyperresponsiveness compared with either drug alone. Although an explanation for this lack of additive effect is uncertain, we speculate that the etiology of AHR is multifactorial and relatively independent of acute, leukotriene-mediated inflammatory events. Although leukotrienes are chemotactic for eosinophils (15, 40), eosinophilic airway inflammation can occur in the absence of AHR (41, 42), and studies using maneuvers to abrogate eosinophilic airway inflammation have shown little effect in attenuating AHR (43, 44). Other inflammatory cells and mediators present in the asthmatic airway are likely to play an important

tenuating AHR (43, 44). Other inflammatory cells and mediators present in the asthmatic airway are likely to play an important role in the pathogenesis of AHR, and we have recently provided evidence to support the notion that a component of AHR results from chronic structural changes that occur as a consequence of allergen-induced acute airway inflammation (45). It may be that budesonide, with broader antiinflammatory actions that potentially affect these chronic structural changes, is likely to have a greater effect in attenuating allergen-induced AHR compared

observed when sputum eosinophils were expressed as absolute cell counts.

DISCUSSION

In this study, we have observed that montelukast treatment significantly reduced the magnitude of allergen-induced early and late asthmatic responses, allergen-induced AHR, and allergen-induced increases in sputum eosinophilia. In contrast, inhaled budesonide treatment had no significant effect on the early asthmatic response, but did, like montelukast, significantly reduce the late asthmatic response, allergen-induced AHR, and allergen-induced increases in sputum eosinophilia. Using the two drugs in combination offered no significant additional benefit in further attenuating the allergen-induced early asthmatic response, allergen-induced AHR, or allergen-induced increases in sputum eosinophilia compared with either drug alone. Furthermore, the combination did not completely abrogate allergeninduced airway eosinophilia.

This study is the first to directly compare the protection afforded by an ICS with that afforded by montelukast against allergen-induced airway responses and inflammation. The results are consistent with the observations of other investigators who have shown that leukotriene modifiers, including montelukast, significantly reduce bronchoconstriction during the early and late asthmatic responses (22, 36–38). However, the study is the first to demonstrate that montelukast has antiinflammatory properties in significantly attenuating allergen-induced sputum eosinophilia. The only other study to evaluate the effect of montelukast on allergen-induced sputum eosinophilia (22) was unable to demonstrate any treatment effect on this outcome. Possible explanations for this difference include the fact that in that study there was a much smaller allergen-induced increase in sputum eosinophilia in the placebo-treated group and that montelukast was given for an insufficient duration.

Montelukast treatment also significantly attenuated allergeninduced AHR. This result is consistent with the findings of a previous study by our group, in which we demonstrated that treatment with the leukotriene modifier, pranlukast, significantly protected against allergen-induced AHR when compared with treatment with placebo (39). In addition, the current study also demonstrates that budesonide afforded significantly greater protection against allergen-induced AHR than did montelukast, whereas the magnitude of the effect on allergen-induced eosinophilia was similar in all three treatment groups.

If, as we have proposed, budesonide and montelukast attenu-

with montelukast, which blocks leukotriene-mediated eosinophilic inflammation.

The lack of clinical benefit from inhaled budesonide, in terms of improvement in FEV₁, between the pretreatment and preallergen phases in the current study, likely relates to the relatively short duration of treatment compared with randomized controlled trials in which 12 months of inhaled budesonide resulted in improvement in lung function (3, 4). Although the current study design was adequately powered (> 90%) to observe a 50% attenuation of the maximum percent late fall in FEV₁ and a 50% attenuation of allergen-induced eosinophilia (46), it was not sufficiently powered to detect clinically significant differences in the bronchoprotective effects of the study medications (47); thus, a change of 0.82 doubling doses in MCh PC₂₀ after budesonide treatment compared with a 0.32 doubling dose change after treatment with the combination of budesonide and montelukast likely reflects the variability of the test.

It is perhaps surprising that both budesonide and montelukast had similar effects on attenuating the allergen-induced sputum eosinophilia, although we have performed several allergen challenge studies in our laboratory, and the results from this study are consistent with previous studies in terms of the magnitudes of attenuation of the early and late asthmatic responses, the allergen-induced AHR, and allergen-induced sputum eosinophilia. A possible explanation for the apparently similar antiinflammatory effects of inhaled budesonide and montelukast may relate to the relatively low dose of inhaled budesonide used in the current study (400 μ g/day). It is apparent that there is a dose-attenuating effect of inhaled steroids on allergen-induced sputum eosinophilia; in a study by our group (9), mometasone furoate in doses of 200 and 800 µg/day attenuated the 7-hour allergen-induced sputum eosinophilia by approximately 60 and 85%, respectively. Similar dose-effects were seen on 24-hour allergen-induced sputum eosinophilia. Similarly, a study by Gauvreau and colleagues (48) showed that 1 week of treatment with 200 µg budesonide twice a day significantly attenuated the allergen-induced sputum eosinophilia at 24 hours after challenge, from $28.8 \pm 4.3\%$ after placebo to $12.6 \pm 2.9\%$ after budesonide treatment. Thus, the magnitude of attenuation was similar to that seen in our study, in which we used the same dose of budesonide for the same duration of treatment. The antiinflammatory effects of montelukast on allergen-induced sputum eosinophilia have only been examined in one previous study (22), as discussed earlier. However, two previous studies in our laboratory (39, 49) showed that leukotriene modifiers attenuated early and late asthmatic responses, as well as allergen-induced AHR, by similar magnitudes as montelukast did in our current study, and we are thus satisfied that the magnitude of attenuation in sputum eosinophils after treatment with montelukast is valid.

Although both budesonide and montelukast treatment, alone and in combination, significantly attenuated allergen-induced airway eosinophilia, none of the three active treatment regimens completely abrogated airway inflammation. This suggests that at least some chemoattractants responsible for recruiting eosinophils into the asthmatic airway act independently of leukotrienemediated, or steroid-sensitive, inflammatory events. Such pathways might also explain why both montelukast and budesonide were similar in inhibiting sputum eosinophilia to equivalent baseline levels. Whereas there has been some recent controversy as to the functional role of eosinophils in the pathogenesis of asthma (43, 50), our results indicate that if in fact, eosinophils do play a central role in asthma, then budesonide and montelukast, either alone or in combination, may be insufficient to optimally control the airway inflammation observed in asthma.

In summary, we have demonstrated that treatment with either budesonide or montelukast had significant antiinflammatory effects when compared with placebo, although treatment with budesonide and montelukast in combination did not provide greater antiinflammatory effects than either drug alone. However, the fact that montelukast was able to attenuate the early asthmatic response and that budesonide and montelukast, either alone or in combination, were able to attenuate the late asthmatic response, allergen-induced AHR, and allergen-induced sputum eosinophilia indicates that these two compounds act on different mediators within the inflammatory pathways that mediate allergen-induced airway inflammation.

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References

- 1. Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001;344:350-362.
- Suissa S, Ernst P, Benayoun S, Baltzan M, Cai B. Low-dose inhaled corticosteroids and the prevention of death from asthma. N Engl J Med 2000;343:332-336.
- Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne PM, Barnes PJ, Ullman A. Effect of inhaled formoterol and budesonide on exacerbations of asthma: formoterol and corticosteroids establishing therapy (FACET) international study group. N Engl J Med 1997;337: 1405–1411.
- O'Byrne PM, Barnes PJ, Rodriguez-Roisin R, Runnerstrom E, Sandstrom T, Svensson K, Tattersfield A. Low-dose inhaled budesonide and formoterol in mild persistent asthma: the OPTIMA randomized trial. *Am J Respir Crit Care Med* 2001;164:1392–1397.
- Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 1992; 145:669–674.
- Laitinen LA, Laitinen A, Haahtela T. A comparative study of the effects of an inhaled corticosteroid, budesonide, and a beta 2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: a randomized, double-blind, parallel-group controlled trial. J Allergy Clin Immunol 1992;90:32–42.
- Busse WW, Brazinsky S, Jacobson K, Stricker W, Schmitt K, Vanden Burgt J, Donnell D, Hannon S, Colice GL. Efficacy response of inhaled beclomethasone dipropionate in asthma is proportional to dose and is improved by formulation with a new propellant. J Allergy Clin Immunol 1999;104:1215–1222.
- Gauvreau GM, Doctor J, Watson RM, Jordana M, O'Byrne PM. Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. *Am J Respir Crit Care Med* 1996;154:1267–1271.
- Inman MD, Watson RM, Rerecich T, Gauvreau GM, Lutsky BN, Stryszak P, O'Byrne PM. Dose-dependent effects of inhaled mometasone furoate on airway function and inflammation after allergen inhalation challenge. Am J Respir Crit Care Med 2001;164:569–574.
- Szefler SJ, Martin RJ, King TS, Boushey HA, Cherniack RM, Chinchilli VM, Craig TJ, Dolovich M, Drazen JM, Fagan JK, et al. Significant variability in response to inhaled corticosteroids for persistent asthma. J Allergy Clin Immunol 2002;109:410–418.
- Dahlen S-E, Hedqvist P, Hammarstrom S, Samuelsson B. Leukotrienes are potent constrictors of human bronchi. *Nature* 1980;286:484–486.
- Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human diseases. N Engl J Med 1990;323:645–655.
- Drazen JM, Israel E, O'Byrne PM. Treatment of asthma with drugs modifying the leukotriene pathway. N Engl J Med 1999;340:197–206.
- Adelroth E, Morris MM, Hargreave FE, O'Byrne PM. Airway responsiveness to leukotrienes C4 and D4 and to methacholine in patients with asthma and normal controls. N Engl J Med 1986;315:480–484.
- Laitinen LA, Laitinen A, Haahtela T. Leukotriene E₄ causes granulocyte infiltration into asthmatic airways. *Lancet* 1993;341:989–990.
- Diamant Z, Hiltermann JT, van Rensen EL, Callenbach PM, Veselic-Charvat M, van der Veen H, Sont JK, Sterk PJ. The effect of inhaled leukotriene D4 and methacholine on sputum cell differentials in asthma. Am J Respir Crit Care Med 1997;155:1247–1253.
- Cohen P, Noveral JP, Bhala A, Nunn SE, Herrick DJ, Grunstein MM. Leukotriene D4 facilitates airway smooth muscle cell proliferation via modulation of the IGF axis. Am J Physiol 1995;269:L151–L157.
- Dahlen S-E, Bjorck J, Hedqvist P. Leukotrienes promote plasma leakage and leukocyte adhesion in post-capillary venules: in vivo effects with

relevance to the acute inflammatory response. Proc Natl Acad Sci USA 1981;78:3887-3891.

- Smith LJ, Greenberger PA, Patterson R, Krell RD, Bernstein PR. The effect of inhaled leukotriene D4 in humans. *Am Rev Respir Dis* 1985; 131:368–372.
- Dworski R, Fitzgerald GA, Oates JA, Sheller JR. Effect of oral prednisone on airway inflammatory mediators in atopic asthma. Am J Respir Crit Care Med 1994;149:953–959.
- Leff JA, Busse WW, Pearlman D, Bronsky EA, Kemp J, Hendeles L, Dockhorn R, Kundu S, Zhang J, Seidenberg BC, et al. Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction. N Engl J Med 1998;339: 147–152.
- 22. Diamant Z, Grootendorst DC, Veselic-Charvat M, Timmers MC, De Smet M, Leff JA, Seidenberg BC, Zwinderman AH, Peszek I, Sterk PJ. The effect of montelukast (MK-0476), a cysteinyl leukotriene receptor antagonist, on allergen-induced airway responses and sputum cell counts in asthma. *Clin Exp Allergy* 1999;29:42–51.
- Pizzichini E, Leff JA, Reiss TF, Hendeles L, Boulet LP, Wei LX, Efthimiadis AE, Zhang J, Hargreave FE. Montelukast reduces airway eosinophilic inflammation in asthma: a randomized, controlled trial. *Eur Respir J* 1999;14:12–18.
- Calhoun WJ, Lavins BJ, Minkwitz MC, Evans R, Gleich GJ, Cohn J. Effect of zafirlukast (Accolate) on cellular mediators of inflammation: bronchoalveolar lavage fluid findings after segmental antigen challenge. Am J Respir Crit Care Med 1998;157:1381–1389.
- Kane GC, Pollice M, Kim CJ, Cohn J, Dworski RT, Murray JJ, Sheller JR, Fish JE, Peters SP. A controlled trial of the effect of the 5lipoxygenase inhibitor, zileuton, on lung inflammation produced by segmental antigen challenge in human beings. J Allergy Clin Immunol 1996;97:646–654.
- Gauvreau GM, Jordana M, Watson RM, Cockroft DW, O'Byrne PM. Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am J Respir Crit Care Med* 1997;156:1738–1745.
- Cockcroft DW, Killian DN, Mellon JJA, Hargreave FE. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977;7:235-243.
- O'Byrne PM, Dolovich J, Hargreave FE. Late asthmatic responses. Am Rev Respir Dis 1987;136:740-751.
- Cockcroft DW, Murdock KY, Kirby J, Hargreave F. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. Am Rev Respir Dis 1987;135:264–267.
- Inman MD, Watson R, Cockcroft DW, Wong BJ, Hargreave FE, O'Byrne PM. Reproducibility of allergen-induced early and late asthmatic responses. J Allergy Clin Immunol 1995;95:1191–1195.
- Spanevello A, Vignola AM, Bonanno A, Confalonieri E, Crimi E, Brusasco V. Effect of methacholine challenge on cellular composition of sputum induction. *Thorax* 1999;54:37–39.
- Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992;47:25–29.
- 33. Pizzichini E, Pizzichini MMM, Efthimiadis A, Evans S, Morris MM, Squillace D, Gleich GJ, Dolovich J, Hargreave FE. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. Am J Respir Crit Care Med 1996;154: 308-317.
- Pizzichini E, Pizzichini MMM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir* J 1996;9:1174–1180.

- Juniper EF, Frith PA, Dunnett C, Cockcroft DW, Hargreave FE. Reproducibility and comparison of responses to inhaled histamine and methacholine. *Thorax* 1978;33:705–710.
- Taylor IK, O'Shaughnessy KM, Fuller RW, Dollery CT. Effect of cysteinyl-leukotriene receptor antagonist ICI 204.219 on allergen-induced bronchoconstriction and airway hyperreactivity in atopic subjects. *Lancet* 1991;337:690–694.
- Rasmussen JB, Eriksson LO, Margolskee DJ, Tagari P, Williams VC, Andersson KE. Leukotriene D4 receptor blockade inhibits the immediate and late bronchoconstrictor responses to inhaled antigen in patients with asthma. J Allergy Clin Immunol 1992;90:193–201.
- Roquet A, Dahlen B, Kumlin M, Ihre E, Anstren G, Binks S, Dahlen SE. Combined antagonism of leukotrienes and histamine produces predominant inhibition of allergen-induced early and late phase airway obstruction in asthmatics. *Am J Respir Crit Care Med* 1997;155:1856– 1863.
- Hamilton A, Faiferman I, Stober P, Watson RM, O'Byrne PM. Pranlukast, a cysteinyl leukotriene receptor antagonist, attenuates allergeninduced early- and late-phase bronchoconstriction and airway hyperresponsiveness in asthmatic subjects. *J Allergy Clin Immunol* 1998;102: 177–183.
- Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. Am J Respir Crit Care Med 2001;164:1495–1500.
- Gibson PG, Dolovich J, Denburg J, Ramsdale EH, Hargreave FE. Chronic cough: eosinophilic bronchitis without asthma. Lancet 1989;1: 1346-1348.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med 2002;346:1699–1705.
- 43. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000;356: 2144–2148.
- 44. Foster PS, Hogan SP, Yang M, Mattes J, Young IG, Matthaei KI, Kumar RK, Mahalingam S, Webb DC. Interleukin-5 and eosinophils as therapeutic targets for asthma. *Trends Mol Med* 2002;8:162–167.
- 45. Leigh R, Ellis R, Wattie J, Southam DS, de Hoogh M, Gauldie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526–535.
- Gauvreau GM, Watson RM, Rerecich TJ, Baswick E, Inman MD, O'Byrne PM. Repeatability of allergen-induced airway inflammation. J Allergy Clin Immunol 1999;104:66–71.
- Inman MD, Hamilton AL, Kerstjens HA, Watson RM, O'Byrne PM. The utility of methacholine airway responsiveness measurements in evaluating anti-asthma drugs. J Allergy Clin Immunol 1998;101:342– 348.
- 48. Gauvreau GM, Wood LJ, Sehmi R, Watson RM, Dorman SC, Schleimer RP, Denburg JA, O'Byrne PM. The effects of inhaled budesonide on circulating eosinophil progenitors and their expression of cytokines after allergen challenge in subjects with atopic asthma. Am J Respir Crit Care Med 2000;162:2139–2144.
- Hamilton AL, Watson RM, Wyile G, O'Byrne PM. Attenuation of early and late phase allergen-induced bronchoconstriction in asthmatic subjects by a 5-lipoxygenase activating protein antagonist, BAYx 1005. *Thorax* 1997;52:348–354.
- O'Byrne PM, Inman MD, Parameswaran K. The trials and tribulations of IL-5, eosinophils, and allergic asthma. J Allergy Clin Immunol 2001; 108:503–508.

CHAPTER 3

DIFFERENT MECHANISMS OF AIRWAY HYPERRESPONSIVENESS IN MICE FOLLOWING BRIEF OR CHRONIC ALLERGEN EXPOSURE

Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM, Inman MD.

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Richard Leigh's contribution:

I am first author on this manuscript, and I was responsible for the collection, management and analysis of study data, and for the writing and subsequent revisions of the final manuscript. The figures, tables and photographs included in this paper are my own work. I am completely familiar with all aspects of data collection, and all those who assisted me did so under my direction. Russ Ellis carried out the histological staining, and supervised the collection and subsequent interpretation of the morphometric data. He also provided valuable help with the figures, tables and photographs, and offered regular feedback on earlier drafts of the manuscript. Jennifer Wattie supervised all the animal handing, including the allergen challenge procedures, and she performed all measurements of airway physiology. David Southam and Meta De Hoog assisted in the collection of morphometric data. Jack Gauldie and Paul O'Byrne provided valuable advice and technical support. Mark Inman provided the academic environment, laboratory facilities, and financial support to carry out the study. As senior author, he was also central to the study design, the supervision of data collection and analysis, and to the supervision of writing and subsequent revisions of the final manuscript.



Firestone Institute for Respiratory Health



April 20, 2004

Christina Shepherd Managing Editor, ATS Editorial Office 61 Broadway, New York, NY 10006-2755

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Dysfunction and Remodeling of the Mouse Airway Persist after Resolution of Acute Allergen-Induced Airway Inflammation

Richard Leigh, Russ Ellis, Jennifer Wattie, David S. Southam, Meta de Hoogh, Jack Gauldie, Paul M. O'Byrne, and Mark D. Inman

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The mechanisms underlying airway hyperresponsiveness remain unclear, although airway inflammation and remodeling are likely important contributing factors. We hypothesized that airway physiology would differ between mice subjected to brief or chronic allergen exposure, and that these differences would be associated with characteristic inflammatory markers and indices of airway remodeling. BALB/c mice were sensitized to ovalbumin and studied at several time points following brief or chronic allergen challenge protocols. By measuring airway responses to methacholine, we demonstrated increases in maximal inducible bronchoconstriction that persisted for 8 wk following either brief or chronic allergen challenge; we also observed increases in airway reactivity, although it was only in chronically challenged mice that these changes persisted beyond the resolution of allergen-induced inflammation. Using airway morphometry, we further demonstrated that increases in maximal bronchoconstriction were associated with increases in airway contractile tissue in both models, and that chronic, but not brief, allergen challenge resulted in subepithelial fibrosis. Our observations that different aspects of sustained airway dysfunction and remodeling persist beyond the resolution of acute inflammatory events support the concept that remodeling occurs as a consequence of allergic airway inflammation, and that these structural changes contribute independently to the persistence of airway hyperresponsiveness.

Asthma is characterized by the presence of variable airflow limitation, airway hyperresponsiveness (AHR) and airway inflammation (1). AHR is present in almost all individuals with asthma, and is characterized by exaggerated airway narrowing following exposure to nonspecific stimuli such as methacholine (MCh), histamine, or exercise (2). Airway responsiveness can be quantified as the provocative dose, or concentration, of a stimulus required to produce a given level of bronchoconstriction (typically a 20% fall in forced expiratory volume in 1 s); as much as 500-fold differences exist between asthmatic and normal individuals (3).

The dysfunction underlying AHR includes hypersensitivity (shift to the left of bronchoconstrictor dose-response

Address correspondence to: Mark D. Inman, M.D., Ph.D., Firestone Institute for Respiratory Health, St. Joseph's Healthcare, 50 Charlton Avenue East, Hamilton, Ontario L8N 4A6 Canada. E-mail: inmanma@mcmaster.ca Abbreviations: airway hyperresponsiveness, AHR; bronchoalveolar lavage,

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BAL; extracellular matrix, ECM; hematoxylin and eosin, H&E; interleukin, IL; intranasal, IN; intraperitoneal, IP; methacholine, MCh; ovalbumin, OVA; periodic acid Schiff, PAS; total respiratory system resistance, R_{RS} ; T helper type 2, Th2; α -smooth muscle actin, α -SMA.

Am. J. Respir. Cell Mol. Biol. Vol. 27, pp. 526–535, 2002 DOI: 10.1165/rcmb.2002-0048OC Internet address: www.atsjournals.org curves), hyperreactivity (increased slope of these curves), and a greater maximum degree of induced bronchoconstriction. However, the pathophysiologic mechanisms underlying these abnormalities remain unclear. T helper type 2 (Th2) inflammation of the airways is believed to be central in the pathogenesis of asthma (4–6), although the exact contribution of airway inflammation to airway dysfunction remains ill-defined (7). While some studies have shown that the extent of airway eosinophilia in asthmatic subjects was related to the degree of their AHR (4, 8), the observation that profound AHR is sustained in asthma, despite prolonged treatment with anti-inflammatory corticosteroids (9–12), suggests that other mechanisms likely account for a major component of AHR.

Evidence suggests that chronic structural changes in the airway, often termed airway remodeling, may be at least in part responsible for sustained AHR (12–16). These changes include thickening of the airway wall, subepithelial fibrosis, hyperplasia and hypertrophy of smooth muscle cells, and hyperplasia of myofibroblasts and goblet cells (17–22). Mathematical modeling studies postulating that both increased muscle mass and, to a lesser extent, airway wall thickening are determinants of AHR (13, 23–24) are supported by airway biopsy evidence that both the degree of smooth muscle thickness and the extent of subepithelial fibrosis relate to the magnitude of AHR in asthma (17, 25–26).

In an attempt to further elucidate potential mechanisms underlying AHR, considerable attention has been paid to mouse models of allergen-induced airway responses (27-36). These models have greatly increased our understanding of the mechanisms underlying transient responses to inhaled allergen, including the role of interleukin (IL)-5 (31, 33–34) in eosinophilic inflammation, and of IL-13 in transient AHR (32, 35–36). However, despite these and other advances, a limitation of these models is that the airway dysfunction is transient, disappearing 14-21 d after allergen exposure, and appears to be related only to acute increases in inflammatory mediators. This is not equivalent to the sustained AHR present in individuals with asthma, and while these models have provided valuable information, they are unlikely to provide a complete description of the mechanisms underlying AHR. We have therefore attempted to further these advances through the development of a model of induced, sustained airway dysfunction in mice.

Our underlying hypothesis is that repeated episodes of allergic inflammation give rise to some of the remodeling changes associated with asthma, which may in turn be assosciated with sustained airway dysfunction. In this study we



Figure 1. Study protocols. Sensitization and challenge protocols used in brief and chronic challenge models. Note that all outcome measurements were made following the final challenge in each protocol.

have addressed the specific questions of (*i*) whether airway physiology differed between mice subjected to either brief or chronic allergen exposure, and (*ii*) whether these differences were associated with characteristic inflammatory markers and indices of airway remodeling.

Materials and Methods

Animals

Female BALB/c mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mice were aged 10 to 12 wk and housed in environmentally controlled, specific pathogen-free conditions for 1 wk prior to study, and for the duration of the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University, and conformed to NIH guidelines for the experimental use of animals (37).

Sensitization

Mice were sensitized with intraperitoneal (IP) ovalbumin (OVA) conjugated to aluminum potassium sulfate injected on Days 1 and 11, and with intranasal (IN) OVA on Day 11. This protocol, including the preparation of OVA, was the same as that used in the past by us (32).

Challenge

Sensitized mice were subjected to either brief or chronic periods of exposure to allergen (Figure 1). Brief exposure involved IN OVA (100- μ g in 25- μ l saline) challenges on Days 18 and 19. Chronic exposure involved six 2-day periods of IN OVA challenges, each separated by 12 d (a total of 12 challenges over a 10 wk period). Control mice were subjected to the same sensitization protocol but received IN saline challenges. Mice were studied at 24 h and 2, 4, and 8 wk after the final exposure to either allergen or saline in both brief and chronic protocols. Separate groups of 10 mice were studied in each protocol and at each end-point, at which time the following outcome measurements were made: (*i*) *in vivo* airway responsiveness to intravenous MCh; (*ii*) total and differential cell counts in bronchoalveolar lavage (BAL) fluid; (*iii*) IL-13 levels in BAL supernatant; and (*iv*) airway morphometry, using a computer-based image analysis system.

Airway Responsiveness

Airway responsiveness was measured based on the response of total respiratory system resistance (R_{RS}) to saline and increasing (10, 33, 100 and 330 µg) intravenous doses of MCh. R_{RS} was a

measured using the flow interrupter technique, modified for use in mice, and described by us and others in detail elsewhere (31, 38). Briefly, mice were anesthetized using 2,2,2-tribromoethanol (Avertin, 240 mg/kg intraperitoneally; Aldrich Chemical Co., Milwaukee, WI). When anesthesia was established, the trachea was exposed and cannulated using a blunted 18-gauge needle. The needle was then attached to a ventilator (RV5; Voltek Enterprises Inc., Toronto, ON, Canada) designed to deliver constant inspiratory flow despite the disturbances in the respiratory system impedance that occur during the MCh challenge. Using the same pattern of ventilation as we have described in the past (31), the response of R_{RS} to increasing doses of intravenous MCh was measured. Rather than obtaining a single index of overall airway responsiveness, we analyzed the resulting R_{RS}-MCh dose response curve, obtaining indices of (i) airway reactivity (slope), (ii) airway sensitivity (lowest dose to produce bronchoconstriction), and (iii) maximal degree of bronchoconstriction (Figure 2). The slope of the relationship was calculated by linear regression between the measured R_{RS} and the \log_{10} transformed MCh dose, using data from the 10, 33 and 100 µg doses. The lowest dose of MCh required to produce bronchoconstriction was determined by calculating the dose at which this regression line intercepted with the postsaline R_{RS} (Figure 2).

Bronchoalveolar Lavage

Following airway physiology measurements, BAL was performed as described previously (31). Briefly, two aliquots of $250-\mu l$ phosphate buffered saline were injected and withdrawn through the



Figure 2. Airway responsiveness methods. Total respiratory system resistance (R_{RS}) was measured in response to increasing doses of intravenous MCh. Using the resulting R_{RS} -MCh dose response curve, indices of airway reactivity (*Slope* R_{RS}), airway sensitivity, or the lowest dose to produce bronchoconstriction (*Break* R_{RS}), and maximal degree of bronchoconstriction (*Max* R_{RS}) were measured.

tracheal cannula. Following centrifugation, the supernatant was stored at -20° C for later IL-13 measurement, and cytocentrifuge slides (Cytospin 3; Shandon Scientific, Sewickley, PA) were prepared and stained with Diff-Quik (Dade Behring Inc., Newark, DE). Differential cell counts were performed on 400 cells by one investigator blind to the experimental conditions. Cells were classified, based on morphologic criteria, as macrophages, neutrophils, lymphocytes, or eosinophils. IL-13 levels in BAL fluid were assessed by enzyme-linked immunosorbent assay (Quantikine, R&D Systems, Minneapolis, MN). Measurements below 7.8 pg/mL are considered by the manufacturer to be below the level of detection.

Lung Histology and Morphometry

The heart and lungs were dissected and removed from the thoracic cavity of each mouse. The lungs were then inflated with 10% formalin to a pressure of 20 cm H_2O and the trachea tied off; both lungs were fixed in formalin for 24 h after which the left lung was isolated, sectioned in half, and the lower half embedded in paraffin. The left lung was oriented so that transverse sections of the first generation bronchus, with the accompanying artery and vein, were

obtained in cross-section (Figure 3). Three- μ m thick transverse sections were cut and stained with hematoxylin and eosin for histological assessment using light microscopy. Additional 3 μ m sections were stained with Masson's Trichrome to demonstrate the presence of extracellular matrix (ECM), and with periodic acid Schiff (PAS) stain to demonstrate the presence of mucin within goblet cells. Further 3- μ m sections were prepared for immunohistochemistry using a monoclonal antibody (Clone 1A4, Dako, Denmark) against α -smooth muscle actin (α -SMA) to identify contractile elements.

Morphometric analysis of the lung sections (10 mice per group), stained as indicated above, was performed using a custom prepared computerized image analysis system (Microscope: Olympus BX40; Camera: Sony 3CCD Power HAD Video Camera; Computer: Pentium III, 700 MHz processor, Windows 98 operating system; Software: Northern Eclipse, version 5 [Empix Imaging Inc., Mississauga, ON, Canada]). Morphometric quantification involved calculating the percent of tissue area that was positively stained within regions of interest (one slide per mouse per stain). Sections of the airway wall that were associated with connective tissue attachments to adjacent vessels were not included in the morpho-



Figure 3. Airway morphometry methods. (A) Transverse section through the left lung (stained with α -SMA) of a first-generation airway (aw) with associated artery (a) and vein (v). The rectangle illustrates a section of airway that is not tethered to a neighboring vessel/ airway and was thus considered suitable for morphometric analysis (enlarged in B–D). (C) The yellow line immediately beneath the epithelium represents that drawn by the user, while the deeper yellow line is that projected by the computer; the computer then isolates the band of tissue in the 20-µm region defined by the two yellow lines. (D) The computer determines the area in this band that corresponds to positive α -SMA staining and expresses this as a percentage of the total band area.

metric analysis of the Masson's Trichrome or α -SMA stain, so as to minimize this area as a potential source of noise. Images of all the remaining first generation airway wall that was identified as being free of intimate contact with the neighboring vessels (Figure 3) were captured into the computer in a blinded fashion by the same operator on each occasion, coded, and stored for later analysis. This process resulted in the analysis of two to three images per slide per mouse.

For sections stained with Masson's Trichrome and α -SMA, the region of interest was a 20µm band immediately beneath the epithelium (this thickness was chosen because it was considered to include the ECM and contractile elements associated with the airways) (Figure 3). Analysis of each stored image was initiated with the operator drawing a line along the basal border of the airway epithelium using a digital pen mouse and writing tablet. This line did not cover any positively stained tissue associated with or beneath the airway epithelium. Thereafter, the macro software application built into the Northern Eclipse Software projected a second line 20 µm beneath the first line. In the case of sections stained with PAS, the operator added the second line along the inner border of the epithelium using the pen mouse, and the region of interest became the area between the two lines. The software then calculated the area between these lines, and regions within this area that were positively stained were identified based on previously determined settings of hue, saturation, and value, used for Masson's Trichrome and α -SMA stained sections, or red, blue, green, used for PAS stained sections. These hue, saturation, and value or red, blue, green limits were identified subjectively prior to any analysis as being able to identify most of the positively stained tissue with minimal identification of negatively stained tissue. Where several separate airway bands of airway wall were analyzed from a single mouse for a particular stain, the final score for that mouse was a weighted mean, where each band was weighted in proportion to its total area. Quantification of peribronchial eosinophilia in airway tissue was achieved through counting the numbers of these cells in the 50-µm region beneath the epithelium of the first generation airway in hematoxylin and eosin stained sections. Cells were expressed as number per mm².

Statistical Analysis

Reported values are expressed as mean and SEM. Comparisons between saline control mice and mice receiving either brief or chronic allergen exposure, with respect to airway reactivity (slope of the R_{RS} log-transformed MCh dose–response curve), maximal bronchoconstriction (maximal MCh–induced R_{RS}), cell counts and BAL IL-13 measurements, and indices of airway remodeling, were made using ANOVA. *Post-hoc* testing with appropriate corrections for multiple comparisons was performed using Duncan's multiple range test. Similar between-group comparisons were made in mice receiving either brief or chronic allergen exposure. All comparisons were two-tailed, and *P* values < 0.05 were considered to be significant.

Results

Differences in Responses to MCh Following Brief or Chronic Exposure to Allergen

The underlying contributors to airway responsiveness, namely airway reactivity (rate of increase in R_{RS} for a given increase in MCh dose), airway sensitivity (lowest dose of MCh to produce bronchoconstriction), and also the maximum inducible bronchoconstriction (maximum R_{RS}), were measured following brief or chronic allergen challenge. Following brief exposure to allergen, there was a significant increase in the airway reactivity as measured by the slope of the MCh dose response curve (Figure 4) at 24 h compared with the saline control group (P < 0.01). However, this transient increase in airway reactivity was no longer present at 2 wk or beyond. In contrast, mice exposed to chronic allergen challenge had a significant increase in airway reactivity at 24 h compared with the saline control group (P <0.01), and this increase was sustained at 2 wk (P = 0.01), 4 wk (P = 0.04), and 8 wk (P = 0.02) postallergen (Figure 4).

Maximal bronchoconstriction was increased at 24 h (P < 0.01), 2 wk (P = 0.05), and 4 wk (P < 0.01) following brief allergen challenge compared with saline controls (Figure 4). Similarly, maximal bronchoconstriction was also increased at 24 h (P < 0.01), 2 wk (P = 0.01), 4 wk (P < 0.01), and 8 wk (P = 0.02) following chronic exposure to allergen. There was no evidence of brief or chronic allergen-induced changes in airway sensitivity, as indicated by the calculated lowest dose of MCh required to increase R_{RS} compared with saline challenged mice.

Figure 4. Time course of airway function and inflammatory changes following brief or chronic exposure to allergen. Airway function (MCh dose response slope and max R_{RS}) and inflammatory markers (peribronchial eosinophils and BAL IL-13) measured at times following brief or chronic exposure to allergen. * indicates P < 0.05 compared with saline challenged mice.





Persistent Airway Hyperreactivity Independent of Airway Eosinophilia

Following brief allergen exposure, there was a significant increase in the number of peribronchial eosinophils at 24 h

Figure 5. Time course of morphometric changes in the airway following brief or chronic exposure to allergen. Staining as assessed using morphometry for ECM (Masson's Trichrome), contractile elements (α -SMA) in the 20 μ m region beneath epithelium, and mucin (PAS) in the epithelial portion of the airway wall, in mice following brief or chronic exposure to allergen. Data are expressed as the percentage of the region of interest that was positively stained. * indicates P < 0.05 compared with saline-challenged mice.

compared with the saline control group (P < 0.01) (Figure 4). Similarly, there was a significant increase in the number eosinophils at 24 h following chronic exposure to allergen compared with the saline control group (P < 0.01). Peri-



Figure 6. Masson's Trichrome-stained sections of airway wall from briefly challenged or chronically challenged mice. Staining for ECM (green) in the airways in saline control mice in the brief (A) or chronic (B) allergen exposure groups, and in mice at 8 wk following brief (C) or chronic (D) exposure to allergen. Bars indicate 100 μ m. 61
bronchial eosinophils had returned to baseline levels 2 wk following either brief or chronic allergen challenge. Responses of BAL eosinophils were similar, increasing from 0 ± 0 (mean \pm SEM) in saline-challenged mice to 10.92 ± 1.71 and 1.59 ± 0.30 , 24 h after brief or chronic challenge, respectively (P < 0.05). BAL eosinophils remained elevated 2 wk after brief or chronic challenge (1.28 ± 0.24 , and 0.21 ± 0.06 , respectively) (P < 0.05), but no eosinophils were detected 4 wk after either challenge. As we have observed previously, allergen challenge was associated with transient increases in neutrophils and lymphocytes (31). However, these had returned to baseline levels by 4 wk after brief or chronic challenge (data not shown).

Sustained Airway Hyperreactivity Independent of Airway IL-13

Following brief exposure to allergen, BAL IL-13 levels were significantly increased at 24 h compared with saline control mice (P < 0.01) (Figure 4). However by 2 wk and beyond, IL-13 levels had returned to baseline levels. In mice receiving chronic allergen exposure, BAL IL-13 levels were also significantly increased at 24 h compared with saline control mice (P < 0.01). Similarly, in mice exposed to chronic allergen, IL-13 measurements fell to baseline levels by 2 wk and were not significantly different at 4 wk, despite the presence of sustained airway hyperreactivity at these time points. IL-13 levels 24 h postchallenge were significantly greater in brief compared with chronic OVA groups (P < 0.01) (Figure 4).

Chronic Exposure to Allergen Associated with Increased Airway ECM

There was no significant change in the amount of ECM present in mouse airways 24 h, or 2, 4, or 8 wk after brief exposure to allergen compared with saline exposed mice (Figure 5; Figures 6 A and 6C). In contrast, mice chronically exposed to allergen had significantly greater ECM present at 4 and 8 wk compared with saline control mice (P < 0.01) and compared with mice receiving brief allergen challenge at all time points (P < 0.01) (Figure 5; Figures 6B and 6D). Although changes in ECM were observed in airways beyond the 1st generation, they appeared less pronounced.

Brief and Chronic Allergen Exposure Both Associated with Increased Contractile Elements

There were significant increases in the amount of α -SMA staining present at 24 h, and 2, 4, and 8 wk following brief exposure to allergen, compared with the saline control group (P < 0.05) (Figure 5; Figures 7A and 7C). Similarly, there were significant increases in α -SMA staining present at 24 h, and 2, 4, and 8 wk after chronic exposure to allergen compared with the saline control group (P < 0.01) (Figure 5; Figures 7B and 7D). Evidence of increased contractile elements was also evident in airways beyond the first generation. Compared with control mice, the average thickness of the airway wall, measured as the distance between the luminal surface of the epithelium and the juncture between the α -SMA-stained region and lung parenchyma, was significantly increased 4 wks after either brief (28.9 \pm 1.7 µm₆?

versus $16.2 \pm 1.2 \ \mu\text{m}$; P < 0.001) or chronic challenge (33.8 $\pm 2.2 \ \mu\text{m}$ versus 18.9 $\pm 1.4 \ \mu\text{m}$; P < 0.001).

Chronic Exposure to Allergen Associated with a Sustained Increase in Airway Epithelium Mucin Content

PAS staining for mucin was significantly increased in the airway epithelium at 24 h and 2 wk after either brief or chronic exposure to allergen compared with saline control mice (P = 0.02 for brief allergen exposure; P < 0.01 for chronic allergen exposure) (Figure 5 and Figures 8C and 8D). By 4 and 8 wk after brief allergen exposure, the amount of mucin present in the airway epithelium returned to levels observed in saline exposed mice (P > 0.05) (Figure 5 and Figure 8E). In contrast, at 4 and 8 wk after chronic allergen exposure, the amount of mucin present in the airway epithelium returned to levels observed in saline exposed mice (P > 0.05) (Figure 5 and Figure 8E). In contrast, at 4 and 8 wk after chronic allergen exposure, the amount of mucin present in the airway epithelium remained significantly increased compared with saline exposed mice (P < 0.01) (Figure 5 and Figure 8F).

Discussion

Using mouse models of either brief or chronic exposure to allergen, we observed different aspects of sustained airway dysfunction and airway remodeling, both of which persisted beyond the resolution of acute immune-mediated inflammatory events. By measuring airway responses to MCh we have demonstrated increases in the maximal inducible bronchoconstriction (maximal R_{RS}), which persist for at least 8 wk following either brief or chronic allergen challenge. In both models this was associated with increases in contractile tissue in the airway wall. We also observed increases in airway reactivity (rate of increase in respiratory resistance for a given increase in MCh dose); however, it was only in chronically challenged mice that these changes persisted beyond the resolution of allergen-induced inflammation. As subepithelial fibrosis was only observed in mice chronically challenged with allergen, it is tempting to speculate that this aspect of remodeling was responsible for genesis of the sustained airway hyperreactivity observed in these mice. However, this line of reasoning is weakened by the fact that fibrosis was only evident at 4 wk after chronic allergen exposure, although sustained airway hyperreactivity was present at 2 wk.

Other investigators have developed models in which structural airway changes occurred following chronic exposure to allergen (39–41). In those studies, airway dysfunction was observed, but was only reported for the period immediately following the final exposure to allergen. Our observations are fundamentally different in that using both brief and chronic challenge protocols, we have demonstrated sustained maximal bronchoconstriction that persists for at least 8 wk following the final exposure to allergen. Furthermore, using our chronic exposure model, we have observed that sustained airway hyperreactivity persists for at least 8 wk following final exposure to allergen. This sustained airway dysfunction was not present in control mice following either brief or chronic exposure to saline.

Previous models of brief exposure to allergen have demonstrated that the resulting transient airway hyperreactivity, measured either as the increase in inflation pressure at a given MCh dose (35), the dose of MCh required to double airway resistance (36), or enhanced pause measured over



Figure 7. α -SMA-stained sections of airway wall from briefly challenged or chronically challenged mice. Staining for contractile elements (red) in the airways in saline control mice in the brief (A) or chronic (B) allergen exposure groups, and in mice at 8 wk following brief (C) or chronic (D) exposure to allergen. Bars indicate 100 μ m.

a range of MCh doses (32) was dependent on IL-13. This paradigm has again been supported in our study by the observation that mice receiving brief exposure to allergen developed transient airway hyperreactivity that was associated with increased levels of IL-13. The specific mechanisms underlying this transient airway hyperresponsiveness are not yet fully understood; however, factors such as transient allergen-induced inflammatory edema, likely to be present but not measured in our study, could play an important role.

In contrast, we have shown that sustained airway hyperreactivity persisting for at least 8 wk following chronic allergen challenge was not associated with ongoing Th2 inflammatory markers, such as airway eosinophilia or increased IL-13 levels. We hypothesize that the sustained airway dysfunction was a consequence of airway remodeling rather than ongoing immune-mediated inflammatory events. Clearly, however, earlier immune-mediated events, including cellular inflammation and increased levels of Th2 cytokines, are likely to contribute to the pathogenesis of this sustained dysfunction.

Our observation that sustained airway hyperreactivity persists after resolution of acute airway inflammation may be analogous to human asthma, where AHR typically persists despite optimal anti-inflammatory therapy (11). Moreover, our findings support the notion that complete resolution of airway dysfunction in established asthma is unlikely to occur using therapies that target specific immune mediators; this may explain why anti-IL-5 therapy had limited benefit in a recent clinical trial (42). However, it is plausible that therapeutic interventions that specifically target immune mediators may limit the extent of disease progression in asthma, if they are initiated earlier in the pathogenesis of the disease.

We have also demonstrated that chronic, but not brief, allergen exposure was associated with significantly increased amounts of ECM in the subepithelial region of the airway wall, and with increased mucin content within the airway epithelium at 4 and 8 wk after the last allergen challenge. These observations are in agreement with other models of chronic exposure to allergen, and support the concept that repeated inflammatory events may contribute to airway remodeling in asthma (39, 41). Although subepithelial fibrosis in the chronic allergen model was not detected until 4 wk after the final challenge, it is possible that this structural change contributed to the persistence of airway reactivity. The possibility that collagen deposition beneath the layer of smooth muscle may enhance the reac-



Figure 8. PAS stained sections of airway epithelium from brief or chronically challenged mice. Staining for mucin (magenta) in the airway epithelium in saline control mice in the brief (A) or chronic (B) allergen exposure groups, in mice at 2 wk following brief (C) or chronic (D) exposure to allergen, and in mice at 8 wk following brief (E) or chronic (F) exposure to allergen. Bars indicate 100 μ m.

tivity of the airways is consistent with the mathematical model of Wiggs and coworkers (13), in which the same degree of smooth muscle constriction in an airway wall thickened by subepithelial collagen deposition resulted in enhanced airway responses to bronchoconstrictor stimuli. Due to the absence of a clear adventitia in the mouse airway, it was not possible to actually measure the thickness of the airway wall.

Interestingly, we did not observe a significant increase in ECM until 4 wk after chronic allergen challenge, despite the fact that mice examined at 24 h and 2 wk had received \underline{A} the full chronic allergen challenge protocol. A possible explanation for this apparent anomaly is that there was an ongoing cellular inflammation at 24 h and 2 wk, as indicated by tissue and BAL eosinophil numbers. Although it is possible that this cellular inflammation simply interfered with the morphometric quantification of ECM, this seems unlikely given that there was no visible evidence of increased Masson's trichrome staining at these time points. A more intriguing hypothesis is that the ongoing cellular inflammation somehow interfered with the deposition of ECM. This is supported by evidence that inflammatory cells are a rich source of matrix metalloproteases (43–44) and may contribute to ongoing clearance of newly formed ECM. Thus, we hypothesize that excessive deposition of ECM was only able to occur once the inflammatory cell infiltrate had resolved between 2 and 4 wk after final exposure to allergen. Our observations illustrate the potential complexity in the relationship between episodic allergic inflammation and fibrotic airway remodeling. Understanding these mechanisms may be crucial in determining which components of the allergic response should be addressed in the pharmacologic management of asthma.

The increase in maximal bronchocontriction associated with the presence of increased staining for contractile tissue suggests a possible causal relationship between increased contractile tissue mass and maximal bronchoconstriction. This finding is consistent with the hypothesis developed by those using mathematical modeling techniques, that increased contractile cell mass is the major determinant of airway narrowing in asthma (13, 23). We have not distinguished between myofibroblasts and smooth muscle within the α -SMA-stained contractile tissue, although increases in either of these cells may contribute to increased airway narrowing. Factors other than the amount of contractile tissue may also influence the degree of maximum airway narrowing. Differences in airway wall edema and fibrosis between mice exposed to either brief or chronic allergen challenge would be expected to influence the degree of airway narrowing (41, 45). The fact that there was a fairly constant increase in the maximal narrowing at all time points in both acute and chronically challenged mice suggests that differences between these influences are likely to be small.

In this study we systematically examined cross-sections of the first generation airway of the left lung of each mouse so as to ensure valid comparisons between each study group. The chronic structural changes observed in these airways were also present in smaller airways, suggesting that these changes occurred more peripherally in the bronchial tree.

Levels of BAL and tissue eosinophils and BAL IL-13 measured 24 h after the final allergen challenge in the chronic protocol were markedly reduced compared with the same measurements at this time point in the brief challenge protocol. This likely indicates that some degree of tolerance developed as a result of chronic OVA exposure.

In conclusion, we have demonstrated that both the airway physiology and markers of allergen-induced inflammation and remodeling differ depending on whether mice were subjected to brief or chronic allergen challenge. Sustained airway dysfunction was observed to be present at least 8 wk following the final exposure to allergen, using either brief or chronic challenge protocols. Sustained airway hyperreactivity was only observed in chronically challenged mice. The observations that this airway dysfunction and indices of airway remodeling persist beyond the resolution of acute immune-mediated inflammatory events support the concept that remodeling occurs as a consequence of repeated allergic airway inflammation, and that these structural changes contribute independently to the persistence of AHR.

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References

- Busse, W. W., and R. F. Lemanske, Jr. 2001. Asthma. N. Engl. J. Med. 344: 350–362.
- Cockcroft, D. W. Airway responsiveness. *In:* Asthma. P. J. Barnes, M. M. Grunstein, A. R. Leff, and A. J. Woolcock, editors. Philadelphia, Lippencourt-Raven Publishers; 1997, p. 1253–1266.
- Inman, M. D., A. L. Hamilton, H. A. Kerstjens, R. M. Watson, and P. M. O'Byrne. 1998. The utility of methacholine airway responsiveness measurements in evaluating anti-asthma drugs. J. Allergy Clin. Immunol. 101:342-348.
- Wardlaw, A. J., S. Dunnette, G. J. Gleich, J. V. Collins, and A. B. Kay. 1988. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am. Rev. Respir. Dis.* 137:62-69.
- Djukanovic, R., W. R. Roche, J. W. Wilson, C. R. Beasley, O. P. Twentyman, R. H. Howarth, and S. T. Holgate. 1990. Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* 142:434–457.
- Bousquet, J., P. Chanez, and J. Y. Lacoste. 1990. Eosinophilic inflammation in asthma. N. Engl. J. Med. 323:1033-1039.
- Lotvall, J., M. Inman, and P. O'Byrne. 1998. Measurement of airway hyperresponsiveness: new considerations. *Thorax* 53:419–424.
- Brusasco, V., E. Crimi, P. Gianiorio, S. Lantero, and G. A. Rossi. 1990. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. J. Appl. Physiol. 69:2209–2214.
- Djukanovic, R., J. W. Wilson, K. M. Britten, S. J. Wilson, A. F. Walls, W. R. Roche, P. H. Howarth, and S. T. Holgate. 1992. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am. Rev. Respir. Dis.* 145:669-674.
- Jeffery, P. K., E. Godfrey, E. Adelroth, F. Nelson, A. Rogers, and S. A. Johansson. 1992. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. *Am. Rev. Respir. Dis.* 145:890–899.
- Sont, J. K., L. N. Willems, E. H. Bel, J. H. van Krieken, J. P. Vandenbroucke, and P. J. Sterk. 1999. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am. J. Respir. Crit. Care Med. 159:1043–1051.
- Boulet, L. P., H. Turcotte, M. Laviolette, F. Naud, M. C. Bernier, S. Martel, and J. Chakir. 2000. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. Am. J. Respir. Crit. Care Med. 162:1308–1313.
- Wiggs, B. R., C. Bosken, P. D. Pare, A. James, and J. C. Hogg. 1992. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* 145:1251–1258.
- Boulet, L. P., J. Chakir, J. Dube, C. Laprise, M. Boutet, and M. Laviolette. 1998. Airway inflammation and structural changes in airway hyperresponsiveness and asthma: an overview. *Can. Respir. J.* 5:16-21.
- Laprise, C., M. Laviolette, M. Boutet, and L. P. Boulet. 1999. Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling. *Eur. Respir. J.* 14:63–73.
- Fish, J. E., and S. P. Peters. 1999. Airway remodeling and persistent airway obstruction in asthma. J. Allergy Clin. Immunol. 104:509-516.
 Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay.
- Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay. 1989. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am. Rev. Respir. Dis.* 140:1745–1753.
- Roche, W. R., R. Beasley, J. H. Williams, and S. T. Holgate. 1989. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1:520-524.
- Carroll, N., J. Elliot, A. Morton, and A. James. 1993. The structure of large and small airways in nonfatal and fatal asthma. *Am. Rev. Respir. Dis.* 147:405–410.
- Elias, J. A., Z. Zhu, G. Chupp, and R. J. Homer. 1999. Airway remodeling in asthma. J. Clin. Invest. 104:1001–1006.
- Busse, W., J. Elias, D. Sheppard, and S. Banks-Schlegel. 1999. Airway remodeling and repair. Am. J. Respir. Crit. Care Med. 160:1035–1042.
- Vignola, A. M., J. Kips, and J. Bousquet. 2000. Tissue remodeling as a feature of persistent asthma. J. Allergy Clin. Immunol. 105:1041–1053.
- Lambert, R. K., B. R. Wiggs, K. Kuwano, J. C. Hogg, and P. D. Pare. 1993. Functional significance of increased airway smooth muscle in asthma and COPD. J. Appl. Physiol. 74:2771–2781.
 Kuwano, K., C. H. Bosken, P. D. Pare, T. R. Bai, B. R. Wiggs, and J. C. Hogg.
- Kuwano, K., C. H. Bosken, P. D. Pare, T. R. Bai, B. R. Wiggs, and J. C. Hogg. 1993. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* 148:1220–1225.
- Boulet, L. P., M. Laviolette, H. Turcotte, A. Cartier, M. Dugas, J. L. Malo, and M. Boutet. 1997. Bronchial subepithelial fibrosis correlates with air-
- 65 way responsiveness to methacholine. Chest 112:45-52.

- Chetta, A., A. Foresi, M. Del Donno, G. Bertorelli, A. Pesci, and D. Olivieri. 1997. Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 111:852–857.
- Renz, H., H. R. Smith, J. E. Henson, B. S. Ray, C. G. Irvin, and E. W. Gelfand. 1992. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. J. Allergy Clin. Immunol. 89:1127-1138.
- Gavett, S. H., D. J. O'Hearn, X. Li, S. K. Huang, F. D. Finkelman, and M. Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. J. Exp. Med. 182:1527-1536.
- Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin-5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J. Exp. Med. 183:109–117.
- Hogan, S. P., K. I. Matthaei, J. M. Young, A. Koskinen, I. G. Young, and P. S. Foster. 1998. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. J. Immunol. 161:1501–1509.
- Inman, M. D., R. Ellis, J. Wattie, J. A. Denburg, and P. M. O'Byrne. 1999. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. Am. J. Respir. Cell Mol. Biol. 21:473–479.
- Webb, D. C., A. N. McKenzie, A. M. Koskinen, M. Yang, J. Mattes, and P. S. Foster. 2000. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. J. Immunol. 165:108–113.
- Wang, J., K. Palmer, J. Lotvall, S. Milan, X. F. Lei, K. I. Matthaei, J. Gauldie, M. D. Inman, M. Jordana, and Z. Xing. 1998. Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia. J. Clin. Invest. 102:1132–1141.
- Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J. Exp. Med. 183:195– 201.

- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258–2261.
- Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261–2263.
- Guide for the Care and Use of Laboratory Animals. Washington, DC.: U.S. Government Printing Office: Revised 1985. NIH Publication No. 86–23.
- Volgyesi, G. A., L. N. Tremblay, P. Webster, N. Zamel, and A. S. Slutsky. 2000. A new ventilator for monitoring lung mechanics in small animals. J. Appl. Physiol. 89:413–421.
- Temelkovski, J., S. P. Hogan, D. P. Shepherd, P. S. Foster, and R. K. Kumar. 1998. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 53:849–856.
- Trifilieff, A., A. El Hashim, and C. Bertrand. 2000. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. Am. J. Physiol. Lung Cell. Mol. Physiol. 279:L1120–L1128.
- Palmans, E., J. C. Kips, and R. A. Pauwels. 2000. Prolonged allergen exposure induces structural airway changes in sensitized rats. Am. J. Respir. Crit. Care Med. 161:627–635.
- 42. Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, T. T. Hansel, S. T. Holgate, P. J. Sterk, and P. J. Barnes. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness, and the late asthmatic response. *Lancet* 356:2144-2148.
- Shapiro, S. D., and R. M. Senior. 1999. Matrix metalloproteinases. Matrix degradation and more. Am. J. Respir. Cell Mol. Biol. 20:1100–1102.
 Ohno, I., H. Ohtani, Y. Nitta, J. Suzuki, H. Hoshi, M. Honma, S. Isoyama,
- Ohno, I., H. Ohtani, Y. Nitta, J. Suzuki, H. Hoshi, M. Honma, S. Isoyama, Y. Tanno, G. Tamura, K. Yamauchi, H. Nagura, and K. Shirato. 1997. Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 16:212–219.
- Brown, R. H., E. A. Zerhouni, and W. Mitzner. 1995. Airway edema potentiates airway reactivity. J. Appl. Physiol. 79:1242–1248.

CHAPTER 4

MORPHOMETRIC ANALYSIS OF MOUSE AIRWAYS AFTER CHRONIC ALLERGEN CHALLENGE

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Richard Leigh's contribution:

I am second author on this manuscript, and I contributed to the planning and design of the experiments, and also participated in the collection, management and analysis of the study data. As such, I am completely familiar with all aspects of data collection contained in this manuscript. I provided regular assistance at all stages of the preparation of the final manuscript. Russ Ellis was primarily responsible for collecting and analyzing the study data, and for the writing and subsequent revisions of the final manuscript. He was also responsible for all the figures, tables and photographs included in this paper. David Southam was involved in the planning and design of the study; he also assisted in the collection of study data and provided regular feedback during the preparation of the final manuscript. Paul O'Byrne provided valuable advice during the preparation of the manuscript. Mark Inman provided the academic environment, laboratory facilities, and financial support to carry out the study. As senior author, he was also central to the study design, to the supervision of data collection and analysis, and to the supervision of the final manuscript.



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Morphometric Analysis of Mouse Airways After Chronic Allergen Challenge

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SUMMARY: Understanding the mechanisms of airway remodeling in chronic allergic conditions such as asthma is increasingly dependent on the use of animal models. Techniques for quantifying structural changes are required that are reproducible and responsive and that can be applied to different staining techniques in both human and animal airway tissues. Here, we characterize a morphometric technique to quantify changes in extracellular matrix and contractile tissue as two indices of airway remodeling in mice. Specific aims were to establish the optimum projection beneath the epithelium to assess remodeling changes and to determine whether such changes are reproducible within different areas of the lung. Finally, based on the variance within measurements, we calculated sample size requirements for research applications of this technique. BALB/c mice were sensitized to ovalbumin and studied after chronic allergen challenge. Lungs were formalin fixed and sectioned were then assayed for extracellular matrix or contractile tissue using morphometric/colorimetric techniques. In this model, the optimum projected distance to measure changes in extracellular matrix or contractile tissue using morphometric dechanges (signal) because of increased irrelevant staining of airway sections and all lung regions studied (p < 0.05). The power of this analysis was such that allergen-induced changes can be reliably (>80% power) detected using 8 to 10 mice. This morphometric technique provides a valid and objective method to assess structural changes in the airways of mice after chronic allergen exposure. (*Lab Invest 2003, 83:1285–1291*).

sthma is characterized by the presence of variable A airflow limitation, airway hyper-responsiveness, and chronic airway inflammation. However, mechanisms underlying the physiologic airway dysfunction remain unclear. Bronchoscopy studies have demonstrated that structural changes are present in asthma (Jeffery et al, 1989), even in the airways of symptomatically mild patients (Chetta et al, 1997). These changes, often grouped under the heading "airway remodeling," include increased smooth muscle (hyperplasia or hypertrophy), thickening of the airway wall (associated with epithelial and goblet cell hyperplasia), myofibroblast hyperplasia, and subepithelial fibrosis (Carroll et al, 1993; Dunnil, 1960; Jeffery et al, 1989, 1992; Roche et al, 1989). Observations that the extent of fibrotic changes are related to the degree of airway eosinophilia (Chetta et al, 1996) and an increase in patients with allergic rhinitis who progress to asthma (Boulet et al. 2000) support the concept that at least some of the remodeling changes are progressive in response to an underlying inflammatory process. Furthermore, findings that the extent of fibrotic changes

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are related to airway hyperresponsiveness (Boulet et al, 1997; Jeffery et al, 1989) and asthma symptoms (Chetta et al, 1997) support the concept that these structural changes play a role in the underlying airway dysfunction in asthma.

Observations that indices of remodeling are present even in newly diagnosed asthma (Chetta et al, 1997) have made it difficult to study the development or functional importance of specific airway remodeling events. For this reason, many investigators have turned to animal models, in which periods of chronic allergen challenge have produced various markers of airway remodeling with an associated airway hyperresponsiveness (Bai et al, 1995; Blease et al, 2001; Palmans et al, 2000; Salmon et al, 1999; Temelkovski et al, 1998). Of these models, those based in mice (Blease et al, 2001; Temelkovski et al, 1998) are particularly attractive given the availability of blocking antibodies and gene-deficient and transgenic mice that allow for mechanistic studies. To study the mechanisms of the remodeling processes and the relationship between resulting structural changes and airway dysfunction, it is crucial that methods used to quantify these structural changes are well characterized, reproducible, and responsive. In the case of assaying the extent of subepithelial fibrosis, methods that have been used in the past include subjective description of the extent of peribronchial collagen staining (Blease et al, 2002), semiguantitative description of the same stain (ie, 0-4 scale) (Henderson et al, 2002), quantification of total lung collagen or hydroxyproline (Lee et

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al, 2001; Sime et al, 1997), and morphometric assessment of specific matrix stains (Lee et al, 2001; Leigh et al, 2002). Changes in peribronchial contractile tissue staining have also been assessed using subjective (Yamamoto et al, 2003) and morphometric (Leigh et al, 2002; Moir et al, 2003) methods. Although each of these methods have demonstrated changes in fibrotic or contractile tissue in response to specific interventions, there is minimal information available indicating how these techniques may be used optimally to detect such changes in the mouse airway wall.

The purpose of the current study was to fully characterize a previously used morphometric method (Leigh et al, 2002) for quantifying subepithelial fibrosis and contractile tissue in mice airways after chronic allergen challenge. This technique combined computerized morphometric and colorimetric techniques to quantify the area positive for Masson's Trichrome (MT) stain or α -smooth muscle actin (α SMA) stain within a given depth beneath the epithelium of the first generation airway. The specific hypothesis addressed in this study was that the power of this technique would be influenced by the depth beneath the epithelium that was analyzed for the presence of each specific stain. We also examined whether detection of allergeninduced changes in these measurements was consistent in several lung regions.

Results

The mean airway ratios for major to minor axes for all tissue segments are shown in Table 1. No differences were detected between saline and allergenchallenged mice (p > 0.05) nor were there differences detected in the ratios between the superior and inferior lung segments from the left lung (p > 0.05). The mean ratio of all sections obtained from the right lung (2.72: sp 1.04) was significantly greater than that for all sections obtained from the left lung (1.19: sp 0.11) (p < 0.05).

What is the Optimal Depth Beneath the Airway Epithelium to Assess Allergen-Induced Fibrotic Changes?

Significant differences in percent MT staining were detected between the saline (SAL) and ovalbumin

Table 1. Airway Ratios (Mean and so) of the First Generation Airway for Each Lobe Segment from SAL and OVA Tissue

Group	Lobe	Airway ratio [mean (sd)]
SAL OVA	Right inferior Left superior Left inferior Right inferior Left superior Left inferior	2.46 (1.07) 1.24 (0.11) 1.17 (0.10) 2.98 (1.01) 1.20 (0.13) 1.16 (0.09)

Ratios calculated as maximum diameter (μ m) divided by minimum diameter (μ m).

SAL, saline; OVA, ovalbumin.

(OVA) tissue using expansion values of 10, 20, 30, 40, and 50 μ m beneath the basal epithelium (p < 0.05) but not values of 60 or 70 μm (Fig. 1A). The greatest magnitude of difference between saline and OVAchallenged mice was 5.49 percentage units, observed using a 20-µm expansion. Sample sizes required to detect differences between the SAL and OVA tissue (β = 0.20) were calculated for each expansion value. The smallest sample size required was 8 mice, using an expansion value of 20 μ m, and ranged from 10 to 16 for the other values examined. For the α SMA stain, significant differences were detected using expansion values of 20, 30, and 40 μ m (p < 0.05) but not values of 10, 50, 60, or 70 μ m (Fig. 1B). The greatest magnitude of difference between saline and OVAchallenged mice was 7.78 percentage units, observed using a 20-µm expansion. Sample sizes required to detect differences between the SAL and OVA tissue (β = 0.20) were calculated for each expansion value. The smallest sample size required was 10 mice, using an expansion value of 20 μ m, and ranged from 14 to 28 for the other values examined.

Are Allergen-Induced Changes in Lung Structure Detected Within Different Lobes or Regions of the Lung?

Significant differences in percent MT staining between SAL and OVA tissue, using a 20- μ m expansion, were detected in each of the three lung segments we studied (p < 0.05) (Fig. 2). The magnitude of difference was larger in the left lung samples (5.17–5.49 percentage units) compared with the right lung samples (4.41



Figure 1.

Quantification of the extent of Masson's Trichrome (MT) staining (A) or α -smooth muscle actin staining (α SMA) (B) in SAL- and OVA-challenged lung tissue when the projected distance from the basement membrane is increased incrementally from 10 to 70 μ m. Sample sizes N ($\beta = 0.20$) were calculated as the number of mice required to detect differences between the SAL and OVA tissue.



Figure 2.

Quantification of the extent of MT staining (A) or α SMA staining (B) in SAL and OVA-challenged lung tissue in single sections from tissue obtained from the right inferior lobe and from the left lobe bisected into superior and inferior segments. Sample sizes N ($\beta = 0.20$) were calculated as the number of mice required to detect differences between the SAL and OVA tissue.

percentage units). Sample sizes required to detect differences between the SAL and OVA tissue ($\beta = 0.20$) were calculated for each lung segment. Both superior and inferior left lobe segments required a sample size of 8 mice to detect differences, whereas the right inferior lobe required a sample size of 10 mice. For the α SMA stain, significant differences were also detected in each of the three lung segments (p < 0.05) (Fig. 2B). Sample sizes required to detect differences between the SAL and OVA tissue ($\beta = 0.20$) were calculated for each lung segment. The left superior lobe required a sample size of 6 mice, the left inferior lobe required 10 mice, and the right inferior lobe required 7 mice.

There were no differences in parenchymal staining between the SAL and OVA groups for each stain. For MT staining, SAL tissue was 0.45% (SEM 0.12) compared with 0.37% (SEM 0.15) for OVA (p > 0.05). For

 α SMA staining, SAL tissue was 1.51% (SEM 0.11) compared with 2.08% (SEM 0.24) for OVA (p > 0.05).

Discussion

The results of this study indicate that this morphometric method is a feasible, valid, and reliable technique to assess subepithelial fibrosis and contractile tissue remodeling in a murine model of chronic allergeninduced airway inflammation. Here we have demonstrated that the power to detect allergen-induced fibrotic and contractile remodeling is influenced by the depth beneath the epithelium that is analyzed for morphometric changes. Moreover, we have observed that these changes seem to occur uniformly in the middle third of the left lung and are also observed in the right lung. This information should be extremely useful to reduce animal numbers in future studies, and to reduce type II errors, in which allergen-induced remodeling might be missed because of poor sampling techniques.

Validity of a test is determined by its ability to consistently detect a true positive signal. Using our current method in our model of chronic allergen challenge, we were able to detect allergen-induced changes in randomly selected sections from three different regions of the left or right lung. The range of expansion depths at which we were able to detect allergen-induced changes in MT staining ranged from 10 to 50 μ m beneath the airway epithelium; the maximal difference was evident at a depth of 20 μ m. At expansion depths >50 mm, differences between OVA- and SAL-challenged mice were no longer apparent and likely reflect a decrease in the signal to noise ratio caused by the inclusion of underlying parenchymal tissue. To detect aSMA staining differences between saline and allergen-challenged mice required expansion depths of 20, 30, or 40 μ m, with maximal differences also detected at 20 µm. Our results indicate that this morphometric analytic technique is a valid method for assessing remodeling of subepithelial fibrotic and contractile tissue in the airway wall. These observations also reflect a feature of this model, namely that fibrotic and contractile tissue changes are restricted to the peribronchial region and do not include lung parenchyma.

A concern when identifying pathologic changes in airway models of chronic inflammation is where in the lung to best locate these changes. Here, we propose that the first generation airway in the middle third of the left lobe should be used for such investigations. The reason for this recommendation is 2-fold. First, this approach allows the investigator to retain a high degree of objectivity in terms of selecting airways for analysis, while ensuring that the airways being compared within each group are of the same generation. Second, we observed that there were few visually obvious changes in the structure of smaller airways. Clearly this latter observation may be influenced by the method of delivering allergen. Our intranasal delivery method is based on aspiration (Southam et al, 2002), resulting in distribution to proximal airways,

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whereas inhalation delivery methods (Ohkawara et al, 1997) would likely result in more peripheral distribution and correspondingly different pathologic changes than observed here.

Our results indicate that a consistently tangential orientation of the airway was obtained when analyzing the left lung and that allergen-induced changes were similar in this lung when we obtained sections from the extreme superior and inferior borders of the middle third of this lung. This demonstrates a considerable degree of latitude for researchers aiming to detect and quantify allergen-induced remodeling changes in similar models.

For appropriate ethical reasons, funding agencies and institutional review boards increasingly require that investigators demonstrate, a priori, that their studies are sufficiently powered (>80%) to detect the primary outcome of interest. Based on power calculations, the results of our study indicate that using an expansion depth of 20 μ m requires the fewest number of mice (8–10 per group) to detect a significant difference in the degree of subepithelial fibrosis or contractile remodeling between SAL control and OVAchallenged mice.

In conclusion, we have described a technique of airway morphometry that is valid and reliable for quantifying the amount of peribronchial extracellular matrix and contractile tissue in a mouse model of chronic airway inflammation. The technique allows investigators to objectively quantify morphologic changes in mouse airways, thereby minimizing investigator bias associated with many of the more subjective techniques currently in use. Clearly, when authors use challenge models that are substantially different from ours, the depth at which remodeling changes occur may differ. In such cases, we strongly recommend that an approach similar to that described here be used to optimize the potential for detecting allergen-induced effects.

Materials and Methods

Animals

Female BALB/c mice, age 10 weeks at study onset, were housed in specific pathogen-free conditions. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University and conformed to National Institutes of Health guidelines (Washington DCUSGPO, 1985).

Sensitization and Challenge

As in the past, we sensitized mice with intraperitoneal ovalbumin (Sigma, Oakville, Ontario, Canada) conjugated to aluminum potassium sulfate (IP OVA) injected on Days 1 and 11 and intranasal ovalbumin (IN OVA) on Day 11 (Inman et al, 1999). Sensitized mice were subjected to chronic periods of allergen exposure, which involved six 2-day periods of IN OVA challenges, each separated by 12 days (10 weeks total). Control mice were sensitized with OVA but received saline (SAL) challenges. Mice (n = 9 SAL, 10 OVA) were studied at 4 weeks after the final challenge (Fig. 3).

Lung Histologic Examination and Morphometry

Dissected lungs were inflated with 10% buffered formalin to a pressure of 20 cmH₂O, ligated at the trachea, and then both lungs were fixed in formalin for 24 hours. After fixation, the right inferior lobe and the left lobe were isolated and bisected into superior and inferior segments (Fig. 4A). After histologic processing



Figure 4.

A, Depiction of the lobes of a mouse lung after inflation and fixation with formalin. The left lobe and right inferior lobe were bisected. Sections for staining were obtained from the exposed surfaces of the right lobe segments, and from regions corresponding to the extreme inferior and superior borders of the middle third of the left lobe (all sections indicated by *dashed lines*). B, Typical first generation airway from the left lobe with associated artery and vein. Airway images captured for analysis indicated in *boxed regions*.



Figure 3.

Sensitization and chronic challenge protocol. Lung tissues were collected after a 4-week recovery period following the final challenge.

(Leigh et al, 2002), both segments of the right inferior lobe were embedded together in paraffin, whereas the superior and inferior segments of the left lobe were embedded in individual paraffin blocks. All tissues were embedded with the sectioned face down and were orientated to obtain transverse cross-sections of the first generation airway (Fig. 4B). This airway is often macroscopically visible in the left lobe before embedding but less so in the right lobe. For this reason, the left lobe segments were embedded separately and the right inferior lobe segments embedded together to ensure good airway representation.

The embedded tissues from the left lobe were rough cut to approximately half the original size, whereas the right lobe segments were rough cut to only expose a smooth tissue surface. Three-micron-thick transverse sections were then cut and stained with either MT to demonstrate extracellular matrix or aSMA (clone NCL-SMA; Novocastra Laboratories Ltd., Newcastle, United Kingdom) to identify contractile elements. For all tissues, the first generation airway was identified while viewing under a microscope at ×4 magnification (Olympus BX40; Carsen Group Inc., Markham, Ontario, Canada), and the major and minor axis were measured with a customized digital image analysis system (Northern Eclipse, version 6; Empix Imaging Inc., Mississauga, Ontario, Canada) using a digital pen mouse and drawing tablet. The ratio of the diameters (maximum/minimum) was calculated as a reflection of the degree to which the sectioning of the airway was at right angles to its long axis.

Morphometric/colorimetric quantification involved calculating the percentage of defined areas of tissue that were positively stained using the same customized digital image analysis system. While viewing under the microscope at $\times 20$ magnification, images of the entire first generation airway wall that were free of intimate contact with the neighboring vessels (Fig. 5A) were captured into the computer by an investigator blinded to the tissue codes. Analysis was initiated by

tracing a line along the basal border of the epithelium using the digital pen mouse and drawing tablet. This line did not cover any stained tissue of interest. Thereafter, the software macro application projected a second line in a direction away from the center of the airway to create a band of tissue for colorimetric analysis. The width of the band was variable and predetermined by the investigator, allowing for repeated analysis on a single image (Fig. 5B). The software then calculated the percentage of each band that was positively stained, based on previously determined color plane settings. Thus, final percent staining scores were obtained for each mouse for both stains of interest. Analytic approaches were aimed at addressing two primary questions:

1. What is the optimal depth beneath the airway epithelium to assess allergen-induced fibrotic changes?

Bands of tissue, with expansion depths ranging from 10 to 70 μ m measured in 10- μ m increments, were obtained from the inferior segment of the left lung (Fig. 5B). Comparisons were made of percent MT staining or percent α SMA staining between SAL and OVA-challenged mice. To assess which band depth is optimal for performing comparative studies, power calculations were performed on these data, with the results expressed as the number of mice required to demonstrate allergen-induced changes in either stain of interest.

2. Are allergen-induced changes in lung structure detected within different lobes or regions of the lung?

The percent MT stain or percent α SMA stain was compared between SAL and OVA-challenged mice using data obtained from a 20- μ m subepithelial band from the first generation airway of the inferior and superior segments of the left lobe and inferior right lobe. Power calculations were performed on these data, with the results expressed as the number of mice required to demonstrate allergen-induced changes in either stain of interest for each lung section.



Figure 5.

A, Depiction of first generation airway captured for analysis. The airway is associated with an artery and vein. B, The basal border of the epithelium is traced by hand (blue line) then projected by software into the surrounding parenchyma (black lines), a distance determined by the operator. The stain of interest (MT) is green and is quantified by the software as a percentage of the total band area.

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To determine that parenchymal staining was equal between SAL and OVA tissues and did not have a systematic influence on morphometric analysis of the airway, bands of parenchymal tissue from the left inferior lobe for both stains were analyzed. A 20- μ m band located a distance between 20 and 40 μ m from the border of the epithelium was captured for analysis. This band was composed primarily of parenchyma, and percent staining of MT or α SMA was analyzed from both SAL and OVA groups.

Statistical Analysis

Data on all figures are expressed as mean and SEM. Descriptive data in Table 1 are expressed as mean and sp. All comparisons between saline control mice and mice receiving chronic allergen exposure were made using Student's *t* test. All comparisons were two-tailed assuming equal variance, and *p* values < 0.05 were considered to be significant. Sample-size requirements were based on a Student's *t* test analysis and calculated with an assumed power of 80% and an α of 0.05 as we have done previously (Dahlen et al, 2001).

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References

Bai TR, Wang ZL, Walker B, and Pare PD (1995). Chronic allergic inflammation induces replication of airway smooth muscle cells in vivo in guinea pigs. Chest 107:93S.

Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, and Hogaboam CM (2001). Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. J Immunol 166:5219–5224.

Blease K, Schuh JM, Jakubzick C, Lukacs NW, Kunkel SL, Joshi BH, Puri RK, Kaplan MH, and Hogaboam CM (2002). Stat6-deficient mice develop airway hyperresponsiveness and peribronchial fibrosis during chronic fungal asthma. Am J Pathol 160:481–490.

Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, and Boutet M (1997). Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. Chest 112:45–52.

Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, and Chakir J (2000). Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma: Influence of inhaled corticosteroids. Am J Respir Crit Care Med 162:1308–1313.

Carroll N, Elliot J, and James A (1993). The structure of the large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis 147:405–410.

Chetta A, Foresi A, Del Donno M, Bertorelli G, Pesci A, and Olivieri D (1997). Airways remodeling is a distinctive feature of asthma and is related to severity of disease. Chest 111:852– 857. Chetta A, Foresi A, Del Donno M, Consigli GF, Bertorelli G, Pesci A, Barbee RA, and Olivieri D (1996). Bronchial responsiveness to distilled water and methacholine and its relationship to inflammation and remodeling of the airways in asthma. Am J Respir Crit Care Med 153:910–917.

Dahlen B, O'Byrne PM, Watson RM, Roquet A, Larsen F, and Inman MD (2001). The reproducibility and sample size requirements of exercise-induced bronchoconstriction measurements. Eur Respir J 17:581–588.

Dunnil MS (1960). The pathology of asthma with special reference to changes in the bronchial mucosa. J Clin Pathol 13:27–32.

Henderson WRJ, Tang L, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, and Chi EY (2002). A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. Am J Respir Crit Care Med 165:108–116.

Inman MD, Ellis R, Wattie J, Denburg JA, and O'Byrne PM (1999). Allergen-induced increase in airway responsiveness, airway eosinophilia and bone marrow eosinophil progenitors in mice. Am J Respir Cell Mol Biol 21:473–479.

Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, and Johansson SA (1992). Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma: A quantitative light and electron microscopic study. Am Rev Respir Dis 145:890–899.

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

Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, and Elias JA (2001). Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). J Exp Med 194:809–821.

Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM, and Inman MD (2002). Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. Am J Respir Cell Mol Biol 27:526–535.

Moir LM, Leung SY, Eynott PR, McVicker CG, Ward JP, Chung KF, and Hirst SJ (2003). Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. American J Physiol Lung Cell Mol Physiol 284:L148–L159.

Ohkawara Y, Lei XF, Stampfli MR, Marshall JS, Xing Z, and Jordana M (1997). Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. Am J Respir Cell Mol Biol 16:510–520.

Palmans E, Kips JC, and Pauwels RA (2000). Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 161:627–635.

Roche WR, Beasley R, Williams JH, and Holgate ST (1989). Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1:520-524.

Salmon M, Walsh DA, Koto H, Barnes PJ, and Chung KF (1999). Repeated allergen exposure of sensitized Brown-Norway rats induces airway cell DNA synthesis and remodelling. Eur Respir J 14:633–641. Sime PJ, Xing Z, Graham FL, Csaky KG, and Gauldie J (1997). Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest 100:768–776.

Southam DS, Dolovich M, O'Byrne PM, and Inman MD (2002). Distribution of intranasal instillations in mice: Effects of volume, time, body position, and anesthesia. Am J Physiol Lung Cell Mol Physiol 282:L833–L839.

Temelkovski J, Hogan SP, Shepherd DP, Foster PS, and Kumar RK (1998). An improved murine model of asthma: Selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolized allergen. Thorax 53:849-856. Washington DCUSGPO (1985). Guide for the Care and Use of Laboratory Animals. National Institutes of Health Publication No. 86-23. Washington DC: U.S. Government Printing Office.

Yamamoto Y, Tanaka A, Kanamaru A, Tanaka S, Tsubone H, Atoji Y, and Suzuki Y (2003). Morphology of aging lung in F344/N rat: Alveolar size, connective tissue, and smooth muscle cell markers. Anat Rec 272A:538–547.

CHAPTER 5

TYPE 2 CYTOKINES IN THE PATHOGENESIS OF SUSTAINED AIRWAY DYSFUNCTION AND AIRWAY REMODELING IN MICE

Leigh R, Ellis R, Wattie J, Hirota J, Matthaei KI, Foster PS, O'Byrne PM, Inman MD.

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Richard Leigh's contribution:

I am first author on this manuscript, and I was involved in the planning and design of the experiments, and was responsible for the collection, management and analysis of study data. The writing of the final manuscript, including the preparation of the figures, tables and photographs was solely my responsibility. I am completely familiar with all aspects of data collection, and all those who assisted me did so under my direction. Russ Ellis supervised histological staining and subsequent collection and subsequent interpretation of the morphometric data. He also provided valuable help with the figures, tables and photographs, and offered regular feedback on earlier drafts of the manuscript. Jennifer Wattie supervised all aspects of animal handing, including the allergen challenge procedures; she performed all measurements of airway physiology. Jeremy Hirota assisted in the collection of morphometric data. Klaus Matthaei and Paul Foster provided us with the IL-5 deficient mice, and along with Paul O'Byrne, offered valuable advice during the preparation of the manuscript. Mark Inman provided the academic environment, laboratory facilities, and financial support to carry out the study. As senior author, he was also central to the study design, the supervision of data collection and analysis, and in supervising the writing and subsequent revisions of the final manuscript.



Firestone Institute for Respiratory Health



April 20, 2004

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Leigh R, Ellis R, Wattie J, Hirota J, Matthaei KI, Foster PS, O'Byrne PM, Inman MD. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. Am J Respir Crit Care Med. 2004; 169: 860-867. A photocopy of the article is enclosed for your reference.

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Type 2 Cytokines in the Pathogenesis of Sustained Airway Dysfunction and Airway Remodeling in Mice

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The mechanisms underlying airway hyperresponsiveness remain unclear, although airway inflammation and remodeling likely play important roles. We have observed sustained airway hyperreactivity and airway remodeling occurring in mice after chronic allergen exposure and persisting beyond resolution of allergen-induced inflammation. The aim of this study was to delineate mechanisms involved in allergen-induced airway hyperreactivity and airway remodeling and to examine evidence for a causal association between airway remodeling and sustained airway hyperreactivity. Wild-type (WT) and interleukin (IL)-4-, IL-5-, and IL-13-deficient (-/-) mice were sensitized and studied 4 weeks after chronic allergen exposure. By measuring airway responsiveness and airway morphometry, we demonstrated that WT mice developed sustained airway hyperreactivity and aspects of airway remodeling after chronic allergen exposure. Both IL-4-/- and IL-13-/- mice were protected from developing sustained airway hyperreactivity and aspects of airway remodeling. In contrast, IL-5^{-/-} mice developed sustained airway hyperreactivity and aspects of airway remodeling similar to that seen in WT mice. Our results confirm that IL-4 and IL-13, but not IL-5, are critical for the development of sustained airway hyperreactivity and airway remodeling after allergen exposure.

Keywords: allergic disease; asthma; interleukin-4; interleukin-5; interleukin-13

Airway hyperresponsiveness (AHR) is a central feature of asthma (1) and is characterized by exaggerated airway narrowing after exposure to nonspecific stimuli such as methacholine (MCh), histamine, or exercise (2). The dysfunction underlying AHR includes hypersensitivity (shift to the left of bronchoconstrictor dose-response curves), hyperreactivity (increased slope of these curves), and a greater maximum degree of induced bronchoconstriction. However, the mechanisms underlying these pathophysiologic abnormalities remain unclear. Numerous experimental and clinical studies have established that CD4⁺ T cell-mediated inflammation of the airways is central to the pathogenesis of asthma (3-5), and the contributions of acute immune-mediated airway inflammation to the pathogenesis of AHR have been investigated in animal models of allergeninduced airway responses (6-10). These studies confirm that the T helper type 2 cytokines interleukin (IL)-4, IL-5, and IL-13 contribute either directly or indirectly to the mechanisms under-

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lying transient allergen-induced AHR by promoting the differentiation, survival, and function of key allergic effector cells (7–9, 11–19). Although these models have increased our understanding of the mechanisms underlying transient responses to allergen, the observed airway dysfunction is transient and appears to be related only to acute increases in inflammatory mediators. Thus, models of brief allergen exposure do not necessarily provide an accurate experimental model of the sustained AHR present in asthma.

Although some studies report that AHR in asthma is related to the extent of T cell-mediated airway inflammation (20, 21), the observation that profound AHR is sustained in asthma despite prolonged treatment with antiinflammatory corticosteroids (22-25) suggests that other mechanisms likely account for a major component of AHR. Evidence suggests that chronic structural changes in the airway, often termed airway remodeling, may be at least partly responsible for sustained AHR (26-32). These changes include thickening of the airway wall, subepithelial fibrosis, hyperplasia and hypertrophy of smooth muscle cells, and hyperplasia of fibroblasts/myofibroblasts and goblet cells (33-41). We have recently described a model in which airway dysfunction and aspects of airway remodeling develop in mice after chronic exposure to allergen (42). These abnormalities persist for at least 8 weeks after final allergen exposure, well beyond the resolution of acute inflammatory events, and suggest that airway remodeling occurs as a consequence of allergic airway inflammation and that aspects of airway remodeling contribute independently to the ongoing, sustained airway hyperreactivity. There have been other reports describing animal models in which structural airway changes occurred after chronic exposure to allergen (32, 43-46). In these studies, airway dysfunction was observed but was either only reported for the period immediately after the final exposure to allergen (43-46) or was measured at the time of ongoing cellular inflammation (32). Our recent observations are fundamentally different in that we have observed that sustained airway hyperreactivity persists for at least 8 weeks after final exposure to allergen (42).

The purpose of this study was to further delineate the mechanisms underlying allergen-induced sustained airway hyperreactivity and airway remodeling and to examine evidence for a causal association between the presence of ongoing sustained airway hyperreactivity and airway remodeling. Although the requirements for IL-4, IL-5, and IL-13 in initiating IgE-mediated eosinophilic airway inflammation and airway dysfunction are established, their respective roles in initiating sustained AHR are not known. By studying mice deficient for IL-4, IL-5, and IL-13, we aimed to determine whether sustained airway dysfunction and aspects of airway remodeling share common critical mechanistic pathways. Some of the results of these studies have been reported previously in the form of an abstract (47).

METHODS

Animals

Female BALB/c wild-type (WT) mice, aged 10 to 12 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME). Female mice of a BALB/c background of similar age, deficient for the IL-4

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gene (IL-4^{-/-}), IL-5 gene (IL-5^{-/-}) (12), or IL-13 gene (IL-13^{-/-}) (48), were obtained from The Jackson Laboratory, the Australian National University in Canberra, Australia, and from Dr. Andrew McKenzie at the Medical Research Council Laboratory of Molecular Biology, Cambridge, UK, respectively. All gene-deficient mice were developed on either a BALB/c or a C57/Bl6 background (in which case they were backcrossed at least eight generations onto a BALB/c background). Mice were housed in environmentally controlled specific pathogen-free conditions for the duration of the experiments. All procedures were approved by the Animal Research Ethics Board at McMaster University and conformed to the National Institutes of Health guidelines for the experimental use of animals.

Sensitization

Mice were sensitized with intraperitoneal ovalbumin conjugated to aluminum potassium sulfate injected on Days 1 and 11 and intranasal ovalbumin on Day 11, as described by us previously (49).

Challenge

Sensitized mice were subjected to either brief or chronic periods of exposure to allergen, as described by us previously (42) (Figure 1). Mice were studied 24 hours after brief exposure protocols or 4 weeks after the chronic protocols. Separate groups of 10 sensitized mice were subjected to saline or ovalburnin exposure in each protocol, and the following outcome measurements were made: (1) in vivo airway responsiveness to intravenous MCh; (2) total and differential cell counts in bronchoalveolar lavage (BAL) fluid; and (3) airway morphometry, using a computer-based image analysis system.

Airway Responsiveness

The underlying contributors to airway responsiveness were measured on the basis of the response of respiratory system resistance to saline and increasing (10, 33, 100, and 330 μ g/kg) intravenous doses of MCh, as described elsewhere in detail by us and others (42, 49, 50).

Bronchoalveolar Lavage

After airway physiology measurements, BAL was performed, as described previously (49).

Lung Morphometry

Lung tissue was prepared for morphometric analysis, as described by us in detail previously (42, 51). Three-micrometer-thick transverse sections were cut and assessed with the following stains: Congo red for the presence of eosinophils; picrosirius red to demonstrate the presence of collagen; and periodic acid–Schiff to demonstrate the presence of musimunohistochemistry using a monoclonal antibody (Novacastra Laboratories Ltd., Newcastle upon Tyne, UK) against α -smooth muscle actin (α -SMA) to identify contractile elements. Morphometric quantification of the stained lung sections was performed using a customized digital image analysis system (Northern Eclipse; Empix Imaging Inc., Missis-

Brief Protocol

Day 19

sauga, ON, Canada), as described by us previously (42, 51). Analysis of the Congo red-stained tissue involved identifying the first generation airway under a microscope at $\times 20$ magnification and capturing images into the computer by an investigator blinded to the tissue codes for the study groups. Analysis involved drawing a line along the basal border of the epithelium; then, using that line as a reference point, the software identified a 50-µm band of tissue in a basal direction. The numbers of eosinophils within this region were counted manually and the results expressed as cells per square millimeter of airway tissue.

Analysis of picrosirius red-stained (viewed using polarized light microscopy) and α -SMA-stained tissue involved identifying the same first generation airway and capturing images of airway wall that were not adjacent to neighboring vessels. As before, a line was drawn along the basal border of the airway epithelium from which a 20-µm band of tissue was projected in a basal direction. The software then calculated the percentage of each band that was positively stained for the individual stains on the basis of previously determined color plane settings. The final score for each stained section was expressed as a weighted mean, with each result weighted in proportion to the total area examined. Goblet cells were identified as periodic acid-Schiff-positive cells in the epithelium and counted manually and expressed as cells per millimeter length of airway wall.

Statistical Analysis

Reported values are expressed as mean and SEM. Comparisons between saline control mice and mice receiving either brief or chronic allergen exposure with respect to airway reactivity (slope of the respiratory system resistance $[R_{RS}] - \log$ -transformed MCh dose-response curve), maximal bronchoconstriction (maximal MCh-induced R_{RS}), cell counts, and indices of airway remodeling were made using analysis of variance. Similar between-group comparisons were made in mice receiving either brief or chronic allergen exposure. *Post hoc* multiple comparison testing was performed using Duncan's test to assess for significant effects. All comparisons were two tailed, and p values less than 0.05 were considered to be significant.

RESULTS

MCh Airway Responses after Brief or Chronic Exposure to Allergen

After brief exposure to allergen, WT mice exhibited significant increases in airway reactivity and maximal bronchoconstriction, compared with the saline control groups (p < 0.01) (Figures 2A, 3, and 4). Similarly, chronic allergen exposure resulted in a significant and sustained increase in airway reactivity and maximal bronchoconstriction in WT mice at 4 weeks, compared with mice given chronic saline exposure (p < 0.01) (Figures 2B, 3, and 4). It should be noted that the magnitude of allergen-induced changes in airway function differed between the groups of WT mice used as controls for each gene-deficient group. Although



Outcomes

Figure 1. Study protocols. Sensitization and challenge protocols used in brief- and chronic-challenge models. Note that all outcome measurements were made after the final challenge in each protocol.



Figure 2. Airway responsiveness in wild-type (WT) mice after brief or chronic exposure to saline or allergen. Total respiratory system resistance (R_{RS}) was measured in response to increasing doses of intravenous methacholine (MCh) in WT mice either 24 hours after brief exposure to saline or allergen (A) or 4 weeks after chronic exposure to saline or allergen (B). Using the resulting R_{RS} – MCh dose–response curve, indices of airway reactivity (slope R_{RS}) and maximal degree of bronchoconstriction (max R_{RS}) were calculated.

we cannot be certain of the reason for this, it likely reflects the fact that, for logistic reasons, there were differences in the time points at which different groups of mice were studied. All genedeficient mice were studied at the same time as the corresponding WT mice, and as such, statistical comparisons have only been made between each gene-deficient group and their own WT control group.

After brief exposure to allergen, IL- $4^{-/-}$ mice demonstrated significant airway hyperreactivity (p < 0.01) and increased maximum bronchoconstriction (p < 0.05), compared with IL-4^{-/-} mice exposed to saline (Figures 3 and 4). However, after chronic allergen exposure, IL-4^{-/-} mice demonstrated normal airway reactivity and normal maximal bronchoconstriction, similar to that present in IL-4^{-/-} mice that had been chronically exposed to saline (Figures 3 and 4). In contrast to IL-4^{-/-} mice, IL-13^{-/-} mice exposed to brief allergen challenge demonstrated normal airway reactivity and normal maximal bronchoconstriction, similar to that present in both WT and IL-13^{-/-} mice given brief saline challenge (Figures 3 and 4). Like the IL- $4^{-/-}$ mice chronically exposed to allergen, IL-13^{-/-} mice also demonstrated normal airway reactivity and normal maximal bronchoconstriction after chronic allergen exposure, similar in magnitude to that present in WT and IL-13^{-/-} mice chronically exposed to saline (Figures 3 and 4).

Like WT and IL-4^{-/-} mice, but in contrast to IL-13^{-/-} mice, IL-5^{-/-} mice demonstrated significant airway hyperreactivity (p < 0.05) and increased maximum bronchoconstriction (p < 0.01) after brief exposure to allergen, compared with IL-5^{-/-} mice exposed to saline (Figures 3 and 4). After chronic allergen exposure, and in contrast to both IL-4^{-/-} and IL-13^{-/-} mice, IL-5^{-/-} mice demonstrated significant airway hyperreactivity (p < 0.05) and increased maximum bronchoconstriction (p < 0.01) compared with IL-5^{-/-} mice chronically exposed to saline (Figures 3 and 4).

Eosinophilic Airway Response after Brief or Chronic Allergen Exposure

After brief allergen exposure, there was a significant increase in the number of peribronchial eosinophils in WT mice compared with the saline control group (p < 0.01) (Figure 5). Both IL- $4^{-/-}$ IL- $13^{-/-}$ mice had an attenuated eosinophil response in comparison with WT mice but still demonstrated a significantly increased eosinophil response in comparison with saline control mice (p < 0.01) (Figure 5). In contrast, the allergen-induced eosinophil response was completely abrogated in IL-5^{-/-} mice after brief allergen exposure, with levels being similar to those in the saline control mice (Figure 5). There was no evidence of significant peribronchial eosinophilia in any of the WT or the IL- $4^{-/-}$, IL- $13^{-/-}$, or IL- $5^{-/-}$ mice when examined 4 weeks after chronic allergen challenge (Figure 5). The magnitude of BAL eosinophilia in response to brief allergen challenge was similar to that seen in the airway tissue (data not shown). Compared with saline control mice, WT as well as IL-4^{-/-} and IL-13^{-/-} mice developed a robust BAL eosinophilia after brief allergen exposure (p < 0.01). In contrast, IL-5^{-/-} mice had a negligible BAL eosinophil response after brief allergen exposure. No BAL eosinophils were detected in any of the study groups 4 weeks after chronic allergen exposure.

Subepithelial Airway Collagen Deposition after Brief or Chronic Allergen Exposure

There was a significant increase in the amount of collagen deposition present in the airways of WT mice 4 weeks after chronic allergen exposure, compared with saline control mice (p < 0.05) (Figures 6, 7A, and 7B). These changes were not present in IL- $4^{-/-}$ and IL- $13^{-/-}$ mice (Figures 6 and 7C–7F). In contrast, IL- $5^{-/-}$ mice exhibited a significant increase in airway collagen deposition 4 weeks after chronic allergen challenge compared with the saline control mice (p < 0.05); indeed, these changes were of a similar magnitude to those seen in WT mice after chronic exposure to allergen (Figures 6, 7A, 7B, 7G, and 7H).

Airway Epithelium Mucin Changes after Brief or Chronic Allergen Exposure

The number of goblet cells staining positively with periodic acid-Schiff for mucin was significantly increased in the airway epithelium of WT mice at 4 weeks after chronic exposure to allergen, compared with saline control mice (p < 0.01) (Figures 6, 8A, and 8B). These changes were completely abrogated in IL-4^{-/-} and IL-13^{-/-} mice after chronic allergen exposure (Figures 6 and 8C-8F). In contrast, IL-5^{-/-} mice exhibited a significant increase in airway epithelium mucin content 4 weeks after chronic allergen challenge compared with saline-exposed mice at the same time point (p < 0.01). This response was however attenuated in comparison with the increase in the amount of mucin present in the airway epithelium of WT mice after chronic allergen exposure (p < 0.05) (Figures 6, 8G, and 8H).

Airway α -SMA Changes after Brief or Chronic Allergen Exposure

There was a significant increase in the amount of α -SMA staining present in WT mice at 4 weeks after chronic exposure to allergen, compared with saline control mice (p < 0.01) (Figures 6, 9A, and 9B). There were no significant differences in α -SMA staining at 4 weeks in any of the IL-4^{-/-}, IL-13^{-/-}, or IL-5^{-/-} mice after chronic exposure to allergen, compared with their corresponding saline control groups (Figures 6 and 9C-9H). However, com-



Figure 3. Airway reactivity in interleukin (IL)-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice and their corresponding WT control mice after brief or chronic exposure to saline or allergen. Airway reactivity, calculated as the dose–response slope to intravenous MCh, measured 24 hours after brief exposure to saline or allergen and 4 weeks after chronic exposure to saline or allergen in IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice and the corresponding WT control mice. *Indicates p value less than 0.05 compared with saline-challenged mice.

pared with saline-challenged WT mice, all the gene-deficient groups had significantly increased amounts of α -SMA staining present, regardless of whether they were chronically exposed to saline or allergen (p < 0.01) (Figures 6, 9A, 9C, 9E, and 9G).

DISCUSSION

In this study, we have confirmed that WT mice develop sustained airway hyperreactivity and aspects of airway remodeling in response to chronic allergen exposure. However, both IL-4^{-/-} and IL-13^{-/-} mice were protected from developing sustained airway hyperreactivity and the associated aspects of airway remodeling, whereas $IL5^{-/-}$ mice were not completely protected from either of these outcomes. These results are consistent with our underlying hypothesis that sustained hyperreactivity is not observed when aspects of remodeling are prevented.

This is the first study to directly examine the role of IL-4, IL-5,

Figure 4. Maximum bronchoconstriction in IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice and their corresponding WT control mice after brief or chronic exposure to saline or allergen. Maximum airway bronchoconstriction in response to intravenous MCh, measured 24 hours after brief exposure to saline or allergen and 4 weeks after chronic exposure to saline or allergen in IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice and the corresponding WT control mice. *Indicates p value less than 0.05 compared with saline-challenged mice.







Figure 5. Airway tissue eosinophil counts in WT, IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice after brief or chronic exposure to saline or allergen. Airway tissue eosinophil counts (expressed as cells per square millimeter) in the 50-µm region beneath the epithelium in the Congo red–stained sections. *Indicates p value less than 0.05 compared with saline-challenged mice.

and IL-13 in an animal model in which both airway remodeling and sustained airway dysfunction are present. The ongoing sustained airway dysfunction that we have observed in our model is fundamentally different from the airway dysfunction described in reports of other models of chronic allergen or fungal exposure, where airway dysfunction is observed at times when cellular airway inflammation is still marked (32, 43, 45, 46). In our current model,





Figure 7. PSR-stained sections of airway wall from chronically challenged mice. Staining for PSR, viewed using polarized light microscopy, in the airways of WT mice after chronic exposure to saline (*A*) or allergen (*B*), in IL-4^{-/-} mice after chronic exposure to saline (*C*) or allergen (*D*), in IL-13^{-/-} mice after chronic exposure to saline (*E*) or allergen (*F*), and in IL-5^{-/-} mice after chronic exposure to saline (*G*) or allergen (*H*). Bars indicate 50 μ m. These photomicrographs depict the upper limit of allergen-induced effects; the mean differences are illustrated in the bar graphs in Figure 6.

sustained airway dysfunction is present for at least 8 weeks after the final allergen challenge, at a time when acute, immune-mediated inflammatory response has resolved (42), and our current results further strengthen the concept that the sustained airway

Figure 6. Morphometric changes in the airway of WT, $IL-4^{-/-}$, $IL-13^{-/-}$, and $IL-5^{-/-}$ mice after brief or chronic exposure to saline or allergen. Morphometric quantification of collagen (picrosirius red [PSR]), mucin (periodic acid–Schiff [PAS]), and contractile elements (α -smooth muscle actin [α -SMA]) in the airway wall of WT, $IL-4^{-/-}$, $IL-13^{-/-}$, and $IL-5^{-/-}$ mice after chronic exposure to saline or allergen. Data are expressed as the percentage of positively stained tissue in the region of interest in the PSR- and α -SMA–stained sections and as the number of goblet cells staining for mucin (PAS positive), expressed per length (cells per millimeter) of airway wall. *Indicates p value less than 0.05 compared with saline-challenged mice.

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Figure 8. PAS-stained sections of airway wall from chronically challenged mice. Staining for PAS in the airways of WT mice after chronic exposure to saline (*A*) or allergen (*B*), in IL-4^{-/-} mice after chronic exposure to saline (*C*) or allergen (*D*), in IL-13^{-/-} mice after chronic exposure to saline (*E*) or allergen (*F*), and in IL-5^{-/-} mice after chronic exposure to saline (*G*) or allergen (*H*). Bars indicate 25 μ m. These photomicrographs depict the upper limit of allergen-induced effects; the mean differences are illustrated in the bar graphs in Figure 6.



Although an analysis of the cytokine networks that regulate allergen-induced airway remodeling is complicated by both the redundant and multifactorial nature of these molecules, the results of our study are largely consistent with other published reports in the literature. Indices of airway remodeling have been reported to be markedly decreased in mice functionally depleted of CD4⁺ T cells after chronic exposure to allergen (5). These results provide evidence that the limited repertoire of T helper type 2-mediated cytokines, including IL-4, IL-5, and IL-13, is likely to play a central role in the mechanisms underlying the development of airway remodeling. We have shown that IL-4 deficiency does not completely attenuate airway eosinophilia but prevents the development of subepithelial fibrosis and goblet cell hyperplasia, as well the development of sustained airway dysfunction. In contrast to this study, a previous report has shown



Figure 9. α -SMA-stained sections of airway wall from chronically challenged mice. Staining for contractile elements (*red*) in the airways of WT mice after chronic exposure to saline (*A*) or allergen (*B*), in IL-4^{-/-} mice after chronic exposure to saline (*C*) or allergen (*D*), in IL-13^{-/-} mice after chronic exposure to saline (*E*) or allergen (*F*), and in IL-5^{-/-} mice after chronic exposure to saline (*G*) or allergen (*H*). Bars indicate 50 μ m. These photomicrographs depict the upper limit of allergen-induced effects; the mean differences are illustrated in the bar graphs in Figure 6.

that indices of airway remodeling were not prevented in IL-4^{-/-} mice after chronic allergen exposure (52). A subsequent study from the same laboratory using the same mouse model reported that IL-4R $\alpha^{-/-}$ mice had significantly less epithelial hypertrophy and goblet cell hyperplasia (but not subepithelial fibrosis) compared with WT mice (53). These data point to the fact that IL-4 may be important in the development of some aspects of airway remodeling.

Our results strongly support the paradigm that the transient airway hyperreactivity occurring after brief exposure to allergen is dependent on IL-13 (15, 16, 18). However, the results of our study extend that understanding by providing novel evidence that IL-13 is necessary for the development of sustained airway dysfunction. Our findings are also consistent with earlier reports (53) demonstrating that IL-13^{-/-} mice exhibited reduced subepithelial fibrosis and goblet cell hyperplasia after chronic allergen exposure and that overexpression of IL-13 resulted in the development of aspects of airway remodeling (17, 54).

In this study, we have observed that IL-5 is not necessary for the development of key aspects of airway remodeling and the associated airway dysfunction after chronic allergen exposure in this model. Our results confirm the observations of several reports, which have suggested that IL-5 (and by implication, eosinophilic inflammation) is not critical for the development of airway dysfunction or aspects of airway remodeling (52, 55). However, given that there may be differences in eosinophil activation in murine models of allergic airway inflammation and in asthma (56), we should not necessarily conclude that eosinophilic inflammation in asthma does not contribute to functionally important airway remodeling. Furthermore, although sustained airway hyperreactivity was still present in IL-5^{-/-} mice, it appeared to be reduced (although not significantly) in relation to the WT mice. We would therefore caution against concluding that IL-5, or by implication airway eosinophilia, is not involved in the development of sustained airway dysfunction.

On the basis of our findings, it is tempting to postulate that indices of airway remodeling, as well as sustained airway dysfunction, can be prevented using strategies that do not affect eosinophilic inflammation. Indeed, we have observed that both these outcome variables are prevented in the IL- $4^{-/-}$ and the IL- $13^{-/-}$ mice despite the fact that eosinophilic airway inflammation was not completely abrogated 24 hours after a brief allergen challenge. However, we do not have data confirming that eosinophilia in these mice persisted throughout the whole period of chronic allergen challenge; we also cannot state that eosinophilic activity was not affected in these mice. Thus, although we believe that we are justified in concluding that airway remodeling in this model is possible in the absence of eosinophilic inflammation, it is clear that further experiments are warranted to address whether controlling eosinophilia can modulate remodeling of the airway.

Thus, our findings are largely consistent with the published literature and strongly support the hypothesis that IL-4 and IL-13 are necessary for the development of aspects of airway remodeling. Our observations also provide a substantial novel finding in that we have observed a consistent relationship between the blocking effects of these cytokines on aspects of airway remodeling and the development of sustained airway dysfunction. We cannot however postulate any role for these cytokines in the remodeling of airway contractile elements, given that the α -SMA staining was increased in all three gene-deficient groups (IL- $4^{-/-}$, IL- $13^{-/-}$, and IL- $5^{-/-}$), compared with WT mice after chronic saline challenge. Although we have provided no direct evidence of a causal relationship between airway remodeling and sustained dysfunction, the consistent association of these variables suggests that such a relationship exists. Bradford-Hill has proposed a set of formal criteria for evaluating and assigning causality (57). Thus, features that would strengthen the argument for a cause and effect relationship include an appropriate temporal relationship, a strong association between purported cause and effect, the existence of a dose-response relationship, a fall in risk when the purported cause is removed, consistency amongst several studies, biological plausibility, and analogy to similar cause and effect relationships. In our experimental model, we have demonstrated both an appropriate temporal relationship as well as a strong association between purported cause and effect between the requirement of IL-4 and IL-13 for the development of airway remodeling after chronic allergen exposure and the associated development of sustained airway hyperreactivity. We have confirmed that sustained airway dysfunction and different aspects of airway remodeling occur in both WT and IL- $5^{-/-}$ mice after chronic exposure to allergen. However, indices of airway remodeling were not seen in either IL-4^{-/-} or IL-13^{-/-} mice, and these mice were completely protected from developing sustained airway hyperreactivity. In our study, we have not demonstrated the existence of a dose-response relationship between IL-4 and IL-13, the development of aspects of airway remodeling,

and the subsequent presence of sustained airway dysfunction. However, indirect evidence for this comes from other studies in which the dose-dependent overexpression of IL-13 in transgenic mice results in collagen deposition and goblet cell hyperplasia and the development of airway dysfunction (17).

In summary, we have demonstrated that IL-4 and IL-13 genedeficient mice were completely protected from developing aspects of airway remodeling and sustained airway hyperreactivity after chronic allergen exposure. In contrast, and despite the virtual absence of tissue eosinophilia, IL-5 gene-deficient mice developed aspects of airway remodeling and sustained airway hyperreactivity similar to that seen in WT mice after chronic allergen exposure. Our results strongly support the concept that airway remodeling and sustained airway dysfunction occur as a consequence of repeated T helper type 2 immune-mediated airway inflammation. Furthermore, they illustrate that IL-4 and IL-13, but not IL-5, are critical for the development of these phenomena.

Conflict of Interest Statement: R.L. has no declared conflict of interest; R.E. has no declared conflict of interest; J.A.H. has no declared conflict of interest; J.A.H. has no declared conflict of interest; K.I.M. has no declared conflict of interest; P.S.F. has no declared conflict of interest; P.M.O. has no declared conflict of interest; M.D.I. has no declared conflict of interest.

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References

- 1. Busse WW, Lemanske RF. Asthma. N Engl J Med 2001;344:350-362.
- Cockcroft DW. Airway responsiveness. In: Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ, editors. Asthma. Philadelphia: Lippincott-Raven Publishers; 1997. p. 1253–1266.
- Djukanovic R, Roche WR, Wilson JW, Beasley CRW, Twentyman OP, Howarth PH, Holgate ST. Mucosal inflammation in asthma. Am Rev Respir Dis 1990;142:434–457.
- Corrigan CJ, Kay AB. T-cells and eosinophils in the pathogenesis of asthma. *Immunol Today* 1992;113:501–507.
- Foster PS, Yang M, Herbert C, Kumar RK. CD4(+) T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma. *Lab Invest* 2002;82:455–462.
- Renz H, Smith HR, Henson JE, Ray PS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. J Allergy Clin Immunol 1992;89:1127–1138.
- Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, Locksley RM. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J Exp Med 1996;183:109–117.
- Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 1996;183:195–201.
- Hamelmann E, Oshiba A, Schwarze J, Bradley K, Loader J, Larsen GL, Gelfand EW. Allergen-specific IgE and IL-5 are essential for the development of airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 1997;16:674–682.
- Hogan SP, Mould A, Kikutani H, Ramsay AJ, Foster PS. Aeroallergeninduced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. J Clin Invest 1997;99:1329–1339.
- Brusselle GG, Kips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA, Bluethmann H. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy* 1994;24:73–80.
- 12. Kopf M, Brombacher F, Hodgkin PD, Ramsay AJ, Milbourne EA, Dai WJ, Ovington KS, Behm CA, Kohler G, Young IG, et al. IL-5-deficient mice have a developmental defect in CD5(+) B-1 cells and lack eosino-philia but have normal antibody and cytotoxic T cell responses. *Immunity* 1996;4:15–24.
- Gavett SH, O'Hearn DJ, Karp CL, Patel EA, Schofield BH, Finkelman FD, Wills-Karp M. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* 1997; 272:L253–L261.
- Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, Locksley RM. Requirements for allergen-induced

- Wills-Karp M, Luyimbazi MJ, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261–2263.
- Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999;103:779–788.
- Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. J Immunol 2000;165:108–113.
- Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885–889.
- Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma: relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988;137: 62-69.
- Brusasco V, Crimi E, Gianiorio P, Lantero S, Rossi GA. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. *J Appl Physiol* 1990;69:2209–2214.
- Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 1992;145:669-674.
- 23. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, Johansson SA. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma: a quantitative light and electron microscopic study. Am Rev Respir Dis 1992;145:890–899.
- Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment: The AMPUL Study Group. Am J Respir Crit Care Med 1999; 159:1043–1051.
- Boulet LP, Cowie RL, Negro RD, Brett W, Gold M, Marques A, Thorburn WS, Stepner NM, Robson R. Comparison of once- with twicedaily dosing of fluticasone propionate in mild and moderate asthma. *Can Respir J* 2000;7:239–247.
- Wiggs BR, Bosken C, Pare PD, James A, Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1992;145:1251–1258.
- Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. J Appl Physiol 1993;74:2771–2781.
- Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1993;148:1220–1225.
- Boulet LP, Chakir J, Dube J, Laprise C, Boutet M, Laviolette M. Airway inflammation and structural changes in airway hyper-responsiveness and asthma: an overview. *Can Respir J* 1998;5:16–21.
- Laprise C, Laviolette M, Boutet M, Boulet LP. Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling. *Eur Respir J* 1999;14:63–73.
- Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, Chakir J. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma: influence of inhaled corticosteroids. Am J Respir Crit Care Med 2000;162:1308–1313.
- 32. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL, Lukacs NW. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. Am J Pathol 2000;156:723–732.
- 33. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma: an ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989;140:1745–1753.

- Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989;1:520–524.
- Carroll N, Elliot J, Morton A, James AL. The structure of large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis 1993;147: 405–410.
- Hegele RG, Hayashi S, Hogg JC, Pare PD. Mechanisms of airway narrowing and hyperresponsiveness in viral respiratory tract infections. *Am J Respir Crit Care Med* 1995;151:1659–1664.
- Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. J Clin Invest 1999;104:1001–1006.
- James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, Carroll NG. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. *Am J Respir Crit Care Med* 2002;166:1590–1595.
- Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. J Clin Invest 1999;104:1001–1006.
- Busse WW, Elias JA, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. Am J Respir Crit Care Med 1999;160:1035–1042.
- Vignola AM, Kips J, Bousquet J. Tissue remodeling as a feature of persistent asthma. J Allergy Clin Immunol 2000;105:1041–1053.
- Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526–535.
- 43. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 1998;53:849–856.
- Trifilieff A, El Hashim A, Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. Am J Physiol Lung Cell Mol Physiol 2000;279:L1120–L1128.
- Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 2000;161:627-635.
- 46. Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y, Nagai H. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res* 2001; 50:616–624.
- 47. Leigh R, Ellis R, Wattie J, Hirota J, O'Byrne PM, Inman MD. IL-4 and IL-13, but not IL-5 are critical for the development of sustained airway hyperreactivity and airway remodeling following chronic allergen challenge in mice [abstract]. Am J Respir Crit Care Med 2003;167:A494.
- McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, Murray R, Grencis R, McKenzie ANJ. Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 1998;9:423–432.
- Inman MD, Ellis R, Wattie J, Denburg JA, O'Byrne PM. Allergeninduced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. Am J Respir Cell Mol Biol 1999;21:473–479.
- Volgyesi GA, Tremblay LN, Webster P, Zamel N, Slutsky AS. A new ventilator for monitoring lung mechanics in small animals. J Appl Physiol 2000;89:413–421.
- Ellis R, Leigh R, Southam DS, O'Byrne PM, Inman MD. Morphometric analysis of mouse airways following chronic allergen challenge. *Lab Invest* 2003;83:1285–1291.
- Foster PS, Ming Y, Matthei KI, Young IG, Temelkovski J, Kumar RK. Dissociation of inflammatory and epithelial responses in a murine model of chronic asthma. *Lab Invest* 2000;80:655–662.
- Kumar RK, Herbert C, Yang M, Koskinen AM, McKenzie AN, Foster PS. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. *Clin Exp Allergy* 2002;32:1104–1111.
- Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, Noble P, Elias JA. IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 2002;168:2953–2962.
- 55. Yang M, Hogan SP, Henry PJ, Matthaei KI, McKenzie AN, Young IG, Rothenberg ME, Foster PS. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and cotaxin. Am J Respir Cell Mol Biol 2001;25:522–530.
- Persson CGA. Con: mice are not a good model of human airway disease. Am J Respir Crit Care Med 2002;166:6–88.
- Bradford-Hill AB. The environment and disease: association or causation? Proc R Soc Med 1965;58:295–300.

CHAPTER 6

T CELL MEDIATED INFLAMMATION DOES NOT CONTRIBUTE TO THE MAINTENANCE OF AIRWAY DYSGUNCTION IN MICE

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Richard Leigh's contribution:

I am first author on this manuscript, and I was involved in the planning and design of the experiments; I was also responsible for the collection, management and analysis of study data. I am completely familiar with all aspects of data collection, and all those who assisted me did so under my direction. The writing of the final manuscript, including the preparation of the figures, tables and photographs was solely my responsibility. David Southam performed all flow-cytometry analyses. Russ Ellis supervised histological staining and the subsequent collection and interpretation of the morphometric data. He also provided valuable help with the figures, tables and photographs, and offered regular feedback on earlier drafts of the manuscript. Jennifer Wattie supervised all aspects of animal handing, including the allergen challenge procedures; she performed all measurements of airway physiology. Roma Sehmi supervised the performing of the flowcytometry, and the subsequent interpretation of those data. Yonghong Wan provided us with the monoclonal antibodies used in the study, and offered valuable advice during the preparation of the manuscript. Mark Inman provided the academic environment, laboratory facilities, and financial support to carry out the study. As senior author, he was also central to the study design, the supervision of data collection and analysis, and in supervising the writing and subsequent revisions of the final manuscript.

T cell mediated inflammation does not contribute to the maintenance of airway dysfunction in mice.

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Running Title: T cells are not necessary to maintain airway dysfunction

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Abstract

T cell mediated airway inflammation is considered to be critical in the pathogenesis of airway hyperresponsiveness. We have described a mouse model in which chronic allergen exposure results in sustained airway hyperresposiveness (AHR) and aspects of airway remodeling, and here sought to determine whether eliminating CD4⁺ and CD8⁺ cells, at a time when airway remodeling had occurred, would attenuate this sustained AHR. Sensitized BALB/c mice were subjected to either brief or chronic periods of allergen exposure, and studied 24 hours after brief or 4 weeks after chronic allergen exposure. In both models, mice received three treatments with anti-CD4 and CD8 monoclonal antibodies during the 10 days prior to outcome measurements. Outcomes included in vivo airway responsiveness to intravenous methacholine, CD4⁺ and CD8⁺ cell counts of lung and spleen using flow cytometric analysis, and airway morphometry using a computer-based image analysis system. Compared to saline control mice, brief allergen challenge resulted in AHR, which was eliminated by antibody treatment. Chronic allergen challenge resulted in sustained AHR and indices of airway remodeling. This sustained AHR was not reversed by antibody treatment, even though CD4⁺ and CD8⁺ cells were absent in lung and spleen. These results indicate that T cell mediated inflammation is critical for development of airway hyperresponsiveness associated with brief allergen exposure, but is not necessary to maintain sustained AHR.

Keywords: Allergy; lung; T lymphocytes; rodent; bronchial hyperreactivity.

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Introduction

The mechanisms underlying airway hyperresponsiveness (AHR) in asthma are complex and likely to be multifactorial (39). There is however considerable evidence to support a role for T cell mediated airway inflammation in the pathogenesis of AHR (6; 11; 13; 19; 42; 48; 52) and several Th2 cytokines, particularly IL-13, have the potential to modulate AHR in animal models of allergic airway inflammation and AHR (7; 12; 18; 21-23; 27; 38; 53; 55). While these experimental models have greatly increased our understanding of the mechanisms underlying transient responses to inhaled allergen, they have all employed models of acute allergic airway inflammation that have centered on short-term exposure of sensitized rodents to allergen. Thus, despite the undoubted advances that have come from these studies, a relative limitation of these models is that the AHR is transient, disappearing 14-21 days after allergen exposure, and appears to be related only to acute increases in inflammatory mediators. This is not equivalent to the chronic airway inflammation and sustained AHR present in individuals with asthma, and while these experimental models have provided valuable information, they are unlikely to provide a complete description of the mechanisms underlying AHR.

Furthermore, the observation that profound AHR is sustained in asthma, despite prolonged treatment with anti-inflammatory corticosteroids (3; 15; 30; 44) suggests that mechanisms other than acute inflammation likely account for a major component of AHR. Evidence suggests that chronic structural changes in the airway, often termed *airway remodeling*, may be at least partly responsible for sustained AHR (2; 4; 5; 29; 33-35; 51; 54). These changes include thickening of the airway wall, subepithelial fibrosis, hyperplasia

and hypertrophy of smooth muscle cells, and hyperplasia of fibroblasts/myofibroblasts and goblet cells (8; 10; 14; 16; 24; 25; 31; 43; 49). We have recently described a model in which AHR and aspects of airway remodeling develop in mice following chronic exposure to allergen (37). These abnormalities persist for at least 8 weeks following final allergen exposure, well beyond the resolution of acute inflammatory events, and suggest that airway remodeling occurs as a consequence of allergic airway inflammation, and that aspects of airway remodeling contribute independently to the ongoing, sustained airway hyperreactivity.

We now hypothesized that the sustained AHR present in our model is independent of T cell orchestrated acute inflammatory events, but is instead the result of chronic structural airway remodeling. Our underlying hypothesis does not question that the initiating events that ultimately result in this remodeling include antigen-induced, T cell dependent acute airway inflammation, but focused instead on whether the ongoing sustained dysfunction was due to the chronic structural changes rather than T cell dependent acute inflammatory events. The purpose of this study was therefore to explore whether the sustained AHR that occurs following chronic exposure to allergen is independent of ongoing T lymphocyte responses. This observation would provide evidence that airway dysfunction in allergic airway diseases such as asthma, may be dependent on factors other than ongoing immune responses.

To do this, we performed a series of experiments in which we initially sought to confirm what other investigators have already demonstrated, namely that depletion of CD4⁺ and CD8⁺ T cells prevented the development of acute, T cell-mediated airway inflammation

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and associated transient AHR in mice, following a period of brief allergen exposure (20). In the second series of experiments, mice were exposed to our chronic allergen challenge protocol and then, at a later point, treated with anti-CD4⁺ and anti-CD8⁺ monoclonal antibodies (mAb). Consistent with our hypothesis, we expected that T cell depletion, on a background of established airway remodeling, was unlikely to attenuate the sustained AHR observed in the model.

Materials and Methods

Animals: Female BALB/c mice, aged 10 - 12 wk, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mice were housed in environmentally controlled specific pathogen free conditions for 1 week prior to study, and for the duration of the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University, and conformed to NIH guidelines for the experimental use of animals.

Sensitization: Mice were sensitized with intraperitoneal ovalbumin conjugated to aluminum potassium sulphate, as described by us previously (28).

Challenge and treatment intervention: Sensitized mice were subjected to either brief or chronic periods of allergen exposure, as described by us previously (37). Control mice were subjected to the same sensitization protocol but received saline challenges. Allergen and control challenged mice were studied at 24 hours after the final exposure (day 21) in the brief allergen challenge protocol, and at 4 weeks after the final exposure in the chronic allergen

challenge protocol (**Figure 1**). Immunodepletion studies were completed using mAb GK1.5 (anti-CD4; ATCC, Manassas, VA) and 2.43 (anti-CD8; ATCC). Hybridoma ascites fluid (100 μ L) for each mAb was diluted in phosphate buffered saline (total volume, 500 μ L) and administered intraperitoneally on days -10, -7 and -3 prior to the outcome day in each protocol (**Figure 1**). Flow cytometry confirmed that the depletion of CD4⁺ and CD8⁺ T cells was consistently >98%. The following outcome measurements were made; 1) *in vivo* airway responsiveness to intravenous methacholine; 2) total and differential cell counts in bronchoalveolar lavage (BAL) fluid; 3) CD4⁺ and CD8⁺ cell counts using Fluorescence Activated Cell Sorter (FACS) analysis, and 4) airway morphometry using a computer based image analysis system. Separate groups of 10 mice were studied in each treatment arm of each protocol.

Airway Responsiveness:

The underlying contributors to airway responsiveness, namely airway reactivity (slope of increase in total respiratory system resistance (R_{RS}) for a given increase in methacholine (MCh) dose), airway sensitivity (lowest dose of MCh to produce bronchoconstriction) and the maximum inducible bronchoconstriction (maximum R_{RS}) were measured following brief or chronic allergen challenge. These components of airway responsiveness were measured based on the response of R_{RS} to saline and increasing (10, 33, 100 and 330 µg/kg) intravenous doses of MCh (**Figure 2**). R_{RS} was measured using the flow interrupter technique, modified for use in mice, and described in detail elsewhere (28; 37; 50).

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Bronchoalveolar Lavage (BAL): Following airway physiology measurements, BAL was performed as described by us previously (28). Differential cell counts were performed on 400 cells, counted by one investigator blind to the experimental conditions. Cells were classified, based on morphological criteria, as macrophages, mononuclear cells, neutrophils or eosinophils.

FACS Analysis: FACS analysis was performed on cells extracted from lung tissue by enzymatic digestion as described by us previously (56). The viability of the extracted lung cells was >95%. A total of 1 x 10^6 cells underlaid with purified anti-mouse CD16/CD32 mAb were incubated for 30 minutes with saturating amounts of flourescein isothiocyanate– anti-mouse CD4 mAb, Cy-Chrome–anti-mouse CD8a mAb and Phycoerythrin–anti-mouse CD90.2 mAb. Analysis was performed on a Becton Dickinson (Franklin Lakes, NJ) FACScan flow cytometer, using Cellquest and WinMDi software packages (BD Biosciences, Oakville, ON, Canada). CD90.2+CD4+ (CD4+), and CD90.2+CD8a+ (CD8+) stained cells were identified by detection of FL-1 (flourescein isothiocyanate), FL-2 (Phycoerythrin) and FL-3 (CyChrome) and expressed as percentages of T cells (all antibodies: Pharmingen, Mississauga, ON). The numbers of positive cells were calculated by multiplying total cell count obtained after lung digestion.

Lung Histology and Morphometry: The lungs were dissected and processed as described by us in detail previously (37). Three-µm thick transverse sections were cut and assessed with the following stains: hematoxylin and eosin (H&E) for the presence of eosinophils;

picrosirius red (PSR) to demonstrate the presence of collagen, and periodic acid Schiff (PAS) to demonstrate the presence of mucin within goblet cells. Additional sections were prepared for immunohistochemistry using a monoclonal antibody (clone α sm-1, Novacastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom) against α -smooth muscle actin (α -SMA) to identify contractile elements. Morphometric quantification of the stained lung sections was performed using a customized digital image analysis system (Northern Eclipse, Empix Imaging Inc., Mississauga, Canada), as described by us previously (17; 37)

Statistical Analysis:

Reported values are expressed as mean and standard error of the mean (SEM). Comparisons with respect to airway reactivity (slope of the R_{RS} - log transformed MCh dose response curve), maximal bronchoconstriction (maximal MCh induced R_{RS}), cell counts and indices of airway remodeling between saline control mice and mice receiving either brief or chronic allergen exposure, treated with either mAb or diluent, were made using analysis of variance (ANOVA). Post-hoc multiple comparison testing was performed using Duncan's test to assess for significant effects. All comparisons were two-tailed, and p values < 0.05 were considered to be significant.

Results

1. Treatment with anti-CD4 and antiCD8 mAb during brief allergen exposure abrogates allergen-induced airway inflammation and prevents the development of AHR.

Following a period of brief allergen challenge, mice treated with diluent (sham) exhibited a significant increase in total cell counts and eosinophils in BAL when compared to mice exposed to saline and treated with diluent (p<0.001) (Table 1). Concurrent treatment with anti-CD4/anti-CD8 mAb during the period of brief allergen challenge (Figure 1) resulted in significant attenuation of the allergen-induced increase in total cell counts and eosinophils in lavage fluid (p<0.001) (Table 1). The magnitude of BAL eosinophilia in response to brief allergen challenge, and the subsequent attenuation by mAb treatment was similar to that seen in the airway tissue (data not shown).

FACS analysis of lung tissue from these allergen challenged / diluent treated animals demonstrated increased numbers of $CD4^+$ and $CD8^+$ cells compared to saline challenged / diluent treated control mice (**Figure 3**). Treatment with anti-CD4/anti-CD8 mAb resulted in the complete elimination of $CD4^+$ and $CD8^+$ T cells from both lung (**Figure 3**) and spleen tissue (data not shown) in these mice.

Following brief exposure to allergen, mice treated with diluent exhibited significant increases in airway reactivity (p=0.03) and maximal bronchoconstriction (p<0.001), compared to the saline control groups (**Figure 4**). We were also able to confirm, as others have shown before(20), that anti-CD4/anti-CD8 mAb treatment during the period of brief allergen challenge completely abrogated the development of airway hyperreactivity and the

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increased maximal bronchoconstriction present in allergen challenged / diluent treated mice (Figure 4).

2. Chronic allergen exposure results in aspects of airway wall remodeling and sustained *AHR*, which are not attenuated by anti-CD4 and antiCD8 mAb treatment.

As expected, minimal numbers of eosinophils were observed in the BAL fluid of diluent treated (sham) and anti-CD4/anti-CD8 mAb treated mice 4 weeks after chronic allergen exposure, and these values were not significantly different to baseline values observed in saline control mice (**Table 1**). CD4⁺ and CD8⁺ cells were absent from the lungs (**Figure 5**) and spleens (data not shown) of mice treated with anti-CD4/anti-CD8 mAb.

There was a significant increase in the amount of mucin containing, PAS positive goblet cells (Figures 6, 7A and 7B), subepithelial collagen deposition (Figures 6, 7D and 7E) and α -SMA staining (Figures 6, 7G and 7H) in the airways of the diluent treated mice 4 weeks after chronic allergen exposure, compared to saline control mice (p<0.01). As in the brief challenge protocol, mAb treatment was given during the 10 days prior to outcome measurements being made, but the timing of this intervention in the chronic protocol meant that treatment with mAb was only given some 3 weeks after the final allergen challenge (Figure1), at a time when remodeling was likely to have become established (37). Anti-CD4/anti-CD8 mAb treatment had no effect on the amount of mucin present in the airway epithelium (Figures 6 and 7C), the amount of airway subepithelial collagen deposition (Figures 6, and 7F), or in the amount of airway wall contractile tissue staining (Figures 6, and 7I).

Following chronic allergen exposure, diluent treated mice exhibited a significant and sustained increase in airway reactivity (p<0.01) and maximal bronchoconstriction (p<0.01) (**Figure 8**), compared to saline control mice. However, in contrast to our observations in the brief exposure model, treatment with anti-CD4/anti-CD8 mAb after chronic allergen exposure, at a time when airway remodeling was likely to have become established, had no attenuating effect on either this sustained airway hyperreactivity or the sustained increase in maximal bronchoconstriction (**Figure 8**).
Discussion

In this study we have shown that anti-CD4 and anti-CD8 monoclonal antibody treatment of mice during a period of brief allergen exposure resulted in attenuation of T cell mediated airway inflammation and prevented the associated transient AHR. Thus, these observations confirm the critical role of $CD4^+$ and $CD8^+$ T cells in initiating the acute inflammatory events that lead to transient AHR in this model, and are consistent with other published reports, in which T cell depletion has resulted in a reduction of the cellular inflammatory infiltrate and AHR (20; 40).

However, this is the first study to directly examine the role of CD4⁺ and CD8⁺ cells in established, sustained AHR. We have now shown that depleting CD4⁺ and CD8⁺ cells (**Figure 5**), at a time when chronic allergen exposure has already resulted in the establishment of airway remodeling (**Figures 6**), does not attenuate the sustained AHR observed in the model (**Figure 8**). These observations are consistent with our underlying hypothesis, namely that T cell-mediated inflammation is not required to maintain sustained AHR, and further strengthen the concept that the sustained AHR is a consequence of airway remodeling, rather than ongoing cellular inflammation.

Although there have been other reports of animal models in which chronic allergen or fungal exposure has resulted in structural airway changes and AHR (26; 41; 45-47), our model is fundamentally different in that the sustained AHR we observe is present for at least 8 weeks after the final allergen challenge, at a time when acute, immune-mediated inflammatory responses have resolved (37). This is in contrast to the AHR described in the other models of chronic allergen or fungal exposure, where AHR is observed at times when cellular airway inflammation is still marked (26; 41; 45; 46).

We recognize that earlier Th2 immune-mediated inflammatory events are likely to be critical in the initial pathogenesis of functionally important airway remodeling processes. Foster and co-workers have already reported that $CD4^+$ T cell depletion at the time of chronic allergen exposure results in attenuation of AHR and aspects of airway remodeling (19). Their observations indicate that T cells contribute to the remodeling process during a period of chronic allergen exposure. Our study was *not* designed to determine whether T cell depletion had any effect on indices of airway remodeling, but instead examined the effects of depleting $CD4^+$ and $CD8^+$ T cells, at a time when they were still present in lung tissue (**Figure 5**) and when aspects of airway remodeling were already established and sustained AHR was present. Our principal hypothesis related to whether ongoing T cell-mediated inflammation was required to maintain sustained AHR; our *a priori* expectation was that the brief period of T cell depletion used in this study was unlikely to have any attenuating effect on indices of airway remodeling.

T cell depletion in these experiments was approximately 98%, raising the question of how many T cells would have been required in order to maintain AHR under chronic allergen conditions. Obviously, the precise answer to this question is not known. However, we observed that the same degree of T cell depletion in the brief allergen challenge protocol was completely effective at abrogating AHR. We therefore assumed that any ongoing T cell dependent AHR in the chronic model would also have been abrogated by the same antibody treatment protocol.

We elected to treat mice with anti-CD4/anti-CD8 mAb for a relatively brief, 10-day period in this study. While this was sufficient to deplete both CD4⁺ and CD8⁺ cells in lung and spleen tissue and to address our study hypothesis, it also provides an opportunity to speculate on the potential effects of extended anti-CD4/anti-CD8 mAb treatment. We assume that concomitant mAb treatment throughout the period of chronic allergen exposure might have resulted in abrogation of Th2 immune-mediated airway inflammation, with the subsequent attenuation of aspects of airway remodeling and AHR, as has been observed by Foster and colleagues (19). It is perhaps more intriguing to speculate whether prolonged mAb treatment, given after a period of chronic allergen exposure, might have facilitated some resolution of the AHR and associated airway remodeling. Formal testing of this hypothesis has important implications for targeted anti-CD4 or more specific immune based treatments as a potential therapy for asthma, and is clinically relevant, in that the majority of patients with asthma already have aspects of airway remodeling present at the time of clinical presentation (9). If prolonged abrogation of T cell-mediated airway inflammation is unable to attenuate aspects of established airway remodeling and AHR, then it is clear that other approaches will be needed to fully address airway dysfunction in asthma. These may take the form of earlier immune based interventions, at a time before significant airway remodeling has occurred, or intervention with other agents that specifically address the functionally important aspects of airway remodeling. Addressing these issues may also provide a better understanding as to why anti-CD4 mAb treatment (32), as well as anti-IL-5 (36) and anti-IL-4 treatment (1) have provided disappointing results in clinical studies of asthma to date.

In summary, we have demonstrated that depletion of CD4⁺ and CD8⁺ cells during brief allergen challenge, at a time when acute, T-cell mediated airway inflammation is associated with transient AHR, results in significant attenuation of the acute inflammation response, and the prevention of the transient AHR. In contrast, despite the depletion of CD4⁺ and CD8⁺ cells after chronic, repeated allergen exposure at a time when airway remodeling has already become established, has no effect on the sustained AHR present in our model at that time. Our results strongly support the paradigm that the transient airway hyperreactivity, occurring after brief exposure to allergen, is dependent on T cell-mediated airway inflammation. However, our observations extend that understanding by providing substantial novel evidence that T cells are not necessary to maintain established sustained AHR, resulting from chronic allergen exposure.

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References

- 1. Borish LC, Nelson HS, Lanz MJ, Claussen L, Whitmore JB, Agosti JM and Garrison L. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebocontrolled trial. *Am J Respir Crit Care Med* 160: 1816-1823, 1999.
- 2. Boulet LP, Chakir J, Dube J, Laprise C, Boutet M and Laviolette M. Airway Inflammation and structural changes in airway hyper- responsiveness and asthma: an overview. *Can Respir J* 5: 16-21, 1998.
- 3. Boulet LP, Cowie RL, Negro RD, Brett W, Gold M, Marques A, Thorburn WS, Stepner NM and Robson R. Comparison of once- with twice-daily dosing of fluticasone propionate in mild and moderate asthma. *Can Respir J* 7: 239-247, 2000.
- 4. Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL and Boutet M. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* 112: 45-52, 1997.
- 5. Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S and Chakir J. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence Of inhaled corticosteroids. *Am J Respir Crit Care Med* 162: 1308-1313, 2000.
- 6. Brusasco V, Crimi E, Gianiorio P, Lantero S and Rossi GA. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. *J Appl Physiol* 69: 2209-2214, 1990.
- 7. Brusselle GG, Kips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA and Bluethmann H. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy* 24: 73-80, 1994.
- 8. Busse W, Elias J, Sheppard D and Banks-Schlegel S. Airway remodeling and repair. *Am J Respir Crit Care Med* 160: 1035-1042, 1999.
- 9. Busse WW and Lemanske RF, Jr. Asthma. N Engl J Med 344: 350-362, 2001.

- 10. Carroll N, Elliot J, Morton A and James A. The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis* 147: 405-410, 1993.
- 11. Corrigan CJ and Kay AB. T-cells and eosinophils in the pathogenesis of asthma. *Immunol Today* 113: 501-507, 1992.
- 12. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP and Locksley RM. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med* 183: 109-117, 1996.
- 13. Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM and Locksley RM. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Molecular Medicine* 4: 344-355, 1998.
- 14. Davies DE, Wicks J, Powell RM, Puddicombe SM and Holgate ST. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 111: 215-225, 2003.
- 15. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH and Holgate ST. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 145: 669-674, 1992.
- 16. Elias JA, Zhu Z, Chupp G and Homer RJ. Airway remodeling in asthma. *J Clin Invest* 104: 1001-1006, 1999.
- Ellis R, Leigh R, Southam DS, O'Byrne PM and Inman MD. Morphometric Analysis of Mouse Airways Following Chronic Allergen Challenge. *Lab Invest* 83: 1285-1291, 2003.
- 18. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI and Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 183: 195-201, 1996.
- 19. Foster PS, Yang M, Herbert C and Kumar RK. CD4(+) T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma. *Lab Invest* 82: 455-462, 2002.

- 20. Gavett SH, Chen X, Finkelman F and Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 10: 587-593, 1994.
- 21. Gavett SH, O'Hearn DJ, Karp CL, Patel EA, Schofield BH, Finkelman FD and Wills-Karp M. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* 272: L253-L261, 1997.
- 22. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM and Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263, 1998.
- 23. Hamelmann E, Oshiba A, Schwarze J, Bradley K, Loader J, Larsen GL and Gelfand EW. Allergen-specific IgE and IL-5 are essential for the development of airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 16: 674-682, 1997.
- 24. Hamid Q. Airway remodeling in asthma. J Allergy Clin Immunol 111: 1420-1421, 2003.
- 25. Hegele RG, Hayashi S, Hogg JC and Pare PD. Mechanisms of airway narrowing and hyperresponsiveness in viral respiratory tract infections. *Am J Respir Crit Care Med* 151: 1659-1664, 1995.
- 26. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL and Lukacs NW. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. *Am J Pathol* 156: 723-732, 2000.
- 27. Hogan SP, Matthaei KI, Young JM, Koskinen A, Young IG and Foster PS. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. *J Immunol* 161: 1501-1509, 1998.
- 28. Inman MD, Ellis R, Wattie J, Denburg JA and O'Byrne PM. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. *Am J Respir Cell Mol Biol* 21: 473-479, 1999.

- 29. James AL, Pare PD and Hogg JC. The mechanics of airway narrowing in asthma. Am Rev Respir Dis 139: 242-246, 1989.
- 30. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A and Johansson SA. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. *Am Rev Respir Dis* 145: 890-899, 1992.
- 31. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV and Kay AB. Bronchial biopsies in asthma: An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 140: 1745-1753, 1989.
- 32. Kon OM, Sihra BS, Compton CH, Leonard TB, Kay AB and Barnes NC. Randomised, dose-ranging, placebo-controlled study of chimeric antibody to CD4 (keliximab) in chronic severe asthma. *Lancet* 352: 1109-1113, 1998.
- 33. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR and Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 148: 1220-1225, 1993.
- Lambert RK, Wiggs BR, Kuwano K, Hogg JC and Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 74: 2771-2781, 1993.
- 35. Laprise C, Laviolette M, Boutet M and Boulet LP. Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling. *Eur Respir J* 14: 63-73, 1999.
- 36. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ and Barnes PJ. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356: 2144-2148, 2000.
- 37. Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM and Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 27: 526-535, 2002.

- 38. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, Hogan SP, Koskinen A, McKenzie AN, Dent LA, Rothenberg ME, Matthaei KI, Young IG and Foster PS. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp Med* 195: 1433-1444, 2002.
- 39. O'Byrne PM and Inman MD. Airway hyperresponsiveness. Chest 123: 411S-416S, 2003.
- 40. Padrid PA, Mathur M, Li X, Herrmann K, Qin Y, Cattamanchi A, Weinstock J, Elliott D, Sperling AI and Bluestone JA. CTLA41g inhibits airway eosinophilia and hyperresponsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *Am J Respir Cell Mol Biol* 18: 453-462, 1998.
- 41. Palmans E, Kips JC and Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 161: 627-635, 2000.
- 42. Robinson DS, Bentley AM, Hartnell A, Kay AB and Durham SR. Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. *Thorax* 48: 26-32, 1993.
- 43. Roche WR, Beasley R, Williams JH and Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1: 520-524, 1989.
- 44. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP and Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 159: 1043-1051, 1999.
- 45. Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y and Nagai H. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res* 50: 616-624, 2001.

- 46. Temelkovski J, Hogan SP, Shepherd DP, Foster PS and Kumar RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 53: 849-856, 1998.
- 47. Trifilieff A, El Hashim A and Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am J Physiol Lung Cell Mol Physiol* 279: L1120-L1128, 2000.
- 48. Venkayya R, Lam M, Willkom M, Grunig G, Corry DB and Erle DJ. The Th2 Lymphocyte Products IL-4 and IL-13 Rapidly Induce Airway Hyperresponsiveness Through Direct Effects on Resident Airway Cells. *Am J Respir Cell Mol Biol* 26: 202-208, 2002.
- 49. Vignola AM, Mirabella F, Costanzo G, Di Giorgi R, Gjomarkaj M, Bellia V and Bonsignore G. Airway remodeling in asthma. *Chest* 123: 4178-422S, 2003.
- 50. Volgyesi GA, Tremblay LN, Webster P, Zamel N and Slutsky AS. A new ventilator for monitoring lung mechanics in small animals. *J Appl Physiol* 89: 413-421, 2000.
- 51. Wang L, McParland BE and Pare PD. The functional consequences of structural changes in the airways: implications for airway hyperresponsiveness in asthma. *Chest* 123: 356S-362S, 2003.
- 52. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV and Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 137: 62-69, 1988.
- 53. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J and Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol* 165: 108-113, 2000.
- 54. Wiggs BR, Bosken C, Pare PD, James A and Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 145: 1251-1258, 1992.

- 55. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL and Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258-2261, 1998.
- 56. Yoshida M, Leigh R, Matsumoto K, Wattie J, Ellis R, O'Byrne PM and Inman MD. Effect of interferon-gamma on allergic airway responses in interferon-gamma-deficient mice. *Am J Respir Crit Care Med* 166: 451-456, 2002.

Figure Legends

Figure 1: Study protocols.

Sensitization and challenge protocols used in brief and chronic challenge models. Intraperitoneal (IP) sensitization with ovalbumin (OVA) was followed by intranasal (IN) challenge with OVA, while control mice received saline. Note that mAb / diluent treatment was given 10, 7 and 3 days prior to outcome measurements being made in each protocol.

Figure 2: Airway responsiveness methods.

Total respiratory system resistance (R_{RS}) was measured in response to increasing doses of i.v. MCh. Using the resulting R_{RS} -MCh dose response curve, indices of airway reactivity (Slope R_{RS}), airway sensitivity, or the lowest dose to produce bronchoconstriction (Break R_{RS}) and maximal degree of bronchoconstriction (Max R_{RS}) were measured.

Figure 3: Lung CD4⁺ and CD8⁺ T cells following brief exposure to saline or allergen.

Percentage of lung CD4⁺ and CD8⁺ T cells, determined by FACS analysis, following brief challenge with either saline (SAL) or ovalbumin (OVA) and treatment with either diluent (DIL) or anti-CD4/anti-CD8 monoclonoal antibodies (mAb). FACS analysis on lung tissues from 2 non-sensitized, unchallenged, untreated nude mice (Jackson Laboratory, Bar Harbor, ME) was performed to establish baseline levels for both CD4⁺ and CD8⁺ cells.

Figure 4: Airway responses following brief exposure to saline or allergen.

Maximum airway bronchoconstriction and airway reactivity, calculated as the dose response slope to intravenous MCh, measured 24h following brief exposure to saline or allergen. Reactivity (slope of the response) of the OVA mice was greater than that of the SAL and OVA+mAb mice (p<0.05), which were not different from each other (p>0.05).

Figure 5: Lung CD4⁺ and CD8⁺ T cells following chronic exposure to saline or allergen. Percentage of lung CD4⁺ and CD8⁺ T cells, determined by FACS analysis, following chronic challenge with either saline (SAL) or ovalbumin (OVA) and treatment with either diluent (DIL) or anti-CD4/anti-CD8 monoclonoal antibodies (mAb). FACS analysis on lung tissues from 2 non-sensitized, unchallenged, untreated nude mice (Jackson Laboratory, Bar Harbor, ME) was performed to establish baseline levels for both CD4 and CD8 cells.

Figure 6: Morphometric changes in airways of mice following chronic exposure to saline or allergen.

Morphometric quantification of mucin containing goblet cells (GC) (PAS), collagen (PSR), and contractile elements (α -SMA) in the airways of mice following chronic challenge with saline (SAL) or ovalbumin (OVA) and treatment with diluent (DIL) or anti-CD4/anti-CD8 monoclonoal antibodies (mAb). Data are expressed as the number of goblet cells staining for mucin (PAS positive) expressed per length of airway wall (GC/mm), and as the percentage of positively stained tissue in the region of interest in the PSR and α -SMA stained sections.

* indicates p < 0.05 compared to saline challenged mice.

Figure 7: Histological sections of airway wall from chronically challenged mice.

Staining for PAS positive goblet cells (**Panels A-C**), collagen deposition (PSR, viewed using polarized light microscopy) (**Panels D-F**), and contractile elements (α SMA) (**Panels G-I**) in the airways of mice following chronic exposure to saline with diluent treatment (**Panels A, D, G**), allergen with diluent treatment (**Panels B, E, H**), or allergen with mAb treatment (**Panels C, F, I**). Bars indicate 50-µm.

Figure 8: Airway responses following chronic exposure to saline or allergen.

Maximum airway bronchoconstriction and airway reactivity, calculated as the dose response slope to intravenous MCh, measured 4 weeks following chronic exposure to saline or allergen. Airway reactivity of the OVA and OVA+mAb mice was greater than that of the SAL mice (p<0.05) but were not different from each other (p>0.05).

	Brief Challenge			Chronic Challenge		
	SAL/DIL	OVA/DIL	OVA/mAb	SAL/DIL	OVA/DIL	OVA/mAb
Total cell counts (x 10 ⁴ /ml)	17.0	106.8*	54. 1 [†]	21.9	38.7	30.7
Eosinophils (x 10 ⁴ /ml)	0.1	70.2*	19.8 [†]	0.0	0.1	0.0

Table 1: Total and eosinophil cell counts in BAL fluid following brief or chronic exposure to saline (SAL) or ovalbumin (OVA). Mice were either treated with diluent (DIL) or anti-CD4 / anti-CD8 monoclonal antibodies (mAb). [†]indicates p < 0.001 compared to saline challenged / diluent treated mice. ^{*}indicates p < 0.001 compared to ovalbumin challenged / diluent treated mice.

Figure 1

Brief Protocol

Outcomes Day 1 Day 11 Bay 19 & 20 IP OVA IP OVA IN OVA IN A A mAb -10 -7 -3

Chronic Protocol

Outcomes































CHAPTER 7

IS INTERLEUKIN-13 CRITICAL TO MAINTAIN MOUSE AIRWAY DYSFUNCTION FOLLOWING CHRONIC ALLERGEN EXPOSURE?

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Richard Leigh's contribution:

I am first author on this manuscript, and I was involved in the planning and design of the experiments; I was also responsible for the collection, management and analysis of study data. I am completely familiar with all aspects of the data collection, and those who assisted me did so under my direction. The writing of the final manuscript, including the preparation of the figures, tables and photographs was solely my responsibility. Russ Ellis supervised histological staining and the subsequent collection and interpretation of the morphometric data. He also provided valuable help with the figures, tables and photographs, and offered regular feedback on earlier drafts of the manuscript. Jennifer Wattie supervised all aspects of animal handing, including the allergen challenge procedures; she performed all measurements of airway physiology. Debra Donaldson provided us with the anti-IL-13 fusion protein used in the study, and offered valuable advice during the planning of the study. Mark Inman provided the academic environment, laboratory facilities, and financial support to carry out the study. As senior author, he was also central to the study design, the supervision of data collection and analysis, and in supervising the writing and subsequent revisions of the final manuscript.

Is interleukin-13 critical in maintaining airway hyperresponsiveness in mice following chronic allergen exposure?

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Running Head: IL-13 in airway hyperresponsiveness

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Abstract

Interleukin (IL)-13 is regarded as being a central effector in the pathophysiology of airway hyperresponsiveness. We have described a mouse model in which chronic allergen exposure results in sustained airway hyperresponsiveness and aspects of airway remodeling, and here sought to demonstrate that this component of airway hyperresponsiveness is independent of biologically active IL-13. Sensitized mice were subjected to either brief or chronic periods of allergen exposure and studied 24 hours after brief or 4 weeks after chronic allergen inhalation. A soluble murine anti-IL-13 receptor fusion protein, that specifically binds to and neutralizes IL-13, was given daily during the 4 days prior to outcome measurements in both protocols. Outcomes included airway responses to intravenous methacholine, bronchoalveolar lavage cell counts and airway morphometry. Compared to saline control, brief allergen challenge resulted in airway hyperresponsiveness, which was prevented by anti-IL-13 treatment. Chronic allergen challenge resulted in sustained airway hyperresponsiveness and indices of airway remodeling; IL-13 blockade failed to reverse this sustained airway hyperresponsiveness. These results confirm that IL-13 is critical for the development of airway hyperresponsiveness associated with brief allergen exposure, but is not necessary to maintain the sustained airway hyperresponsiveness associated with airway remodeling.

Key words: Allergic disease; asthma; bronchial hyperreactivity; airway inflammation.

Introduction

Experimental animal models point to an essential role for interleukin (IL)-13 in the induction of airway hyperresponsiveness (AHR)¹⁻⁹. The administration of recombinant IL-13 to the airways of naïve mice, in the absence of allergen immunization, induces airway inflammation and AHR^{1,2}. Furthermore, selective blockade of IL-13, performed by the systemic administration of a soluble IL-13 receptor fusion protein, is effective in abrogating allergen-induced AHR in mouse models that involve brief periods of allergen exposure^{1,2}. Transgenic models in which mice constitutively over-express IL-13 in their airways have also provided convincing evidence for the effector role of IL-13 in the pathogenesis of AHR. These mice develop acute and chronic airway inflammation, which is associated with spontaneous increases in airway resistance and airway hyperreactivity to nebulized methacholine³. Based largely on these pre-clinical observations, IL-13 is now widely regarded as being a central mediator in the pathophysiology of AHR¹⁰⁻¹³.

However, the mechanisms underlying AHR in asthma are complex and likely to be multifactorial ¹⁴. We postulate that there are times when patients with stable asthma, but persistent AHR, will not have major ongoing immune events. The observation that profound AHR is sustained in asthma, despite effective treatment with anti-inflammatory corticosteroids ¹⁵⁻¹⁸ suggests that mechanisms other than acute T-helper type 2 (Th2) cell mediated inflammation likely account for a major component of AHR. An increasingly large body of literature supports the paradigm that chronic structural changes in the airway, often termed *airway remodeling*, may be at least partly responsible for sustained AHR ¹⁹⁻²⁷. These changes include airway wall thickening, subepithelial fibrosis, goblet cell metaplasia and hypertrophy and hyperplasia of myocytes, fibroblasts and myofibroblasts ²⁸⁻³³. Although modeling systems based on short-term

exposure of sensitized animals to allergen have greatly increased our understanding of the mechanisms underlying Th2-mediated inflammatory responses, a relative limitation of these models is that they do not adequately account for the chronic structural changes present in asthma. Thus, the associated AHR is transient, disappearing 14-21 days after allergen inhalation, and appears to be related only to Th2-mediated airway inflammation and the activation of Th2 cytokine effector pathways ³⁴. This is not equivalent to the sustained AHR present in individuals with established asthma and thus, while these brief challenge models have provided valuable information, they are unlikely to provide a complete description of the mechanisms underlying AHR.

We have described a model in which sustained AHR and indices of airway remodeling develop in mice following chronic exposure to allergen ³⁴. We believe that both the AHR and much of the remodeling evident in this model are IL-13 dependent, as both are prevented when IL-13 gene-deficient mice are chronically exposed to allergen in this protocol ³⁵. However, the fact that these abnormalities *persist* in wild type mice for at least 8 weeks following final allergen exposure, beyond the resolution of acute inflammatory events, suggest that aspects of airway remodeling contribute independently to the ongoing, sustained airway hyperreactivity ³⁴. We now hypothesized that the sustained airway dysfunction present in our model is no longer dependent on IL-13 effector mechanisms, but is instead associated with chronic structural airway remodeling. The purpose of the present study was to demonstrate that there is an IL-13 independent component of AHR. To do this, we performed a series of experiments in which we initially sought to confirm that blockade of IL-13 with a soluble IL-13 receptor fusion protein prevented the development of the acute, inflammatory associated transient AHR in mice following brief allergen exposure. These experiments served as a positive control to confirm the

neutralizing effects of the anti-IL-13 fusion protein on allergen-induced AHR. In the second series of experiments, mice were exposed to our chronic allergen challenge protocol, and then, *at a later point*, several weeks after chronic challenge, treated with soluble IL-13 receptor fusion protein to block the effect of biologically active IL-13. Consistent with our hypothesis, we expected that IL-13 neutralization at this time was unlikely to attenuate the sustained AHR present in our model. Some of the results of these studies have been previously reported in the form of an abstract ³⁶.

Materials and Methods

Animals: Female BALB/c mice, aged 10-12 weeks, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mice were housed in environmentally controlled specific pathogen free conditions for 1 week prior to study, and for the duration of the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University, and conformed to NIH guidelines for the experimental use of animals.

Sensitization: Mice were sensitized with intraperitoneal ovalbumin conjugated to aluminum potassium sulphate, as described by us previously ³⁷.

Challenge and treatment intervention: Sensitized mice were subjected to either brief or chronic periods of allergen exposure, as described by us previously ³⁴ (Figure 1). Control mice were subjected to the same sensitization protocol but received saline challenges. Allergen and saline challenged mice were studied 24 hours after the final exposure (day 21) in the brief protocol, and 4 weeks after the final exposure in the chronic protocol. Blockade of IL-13 was performed by

intraperitoneal administration of a soluble murine IL-13r α 2-humanIgG fusion protein (sIL-13R α 2.Fc) provided by Wyeth Pharmaceuticals Inc. (Cambridge, MA), which specifically binds to and neutralizes murine IL-13 ³⁸. Control mice were treated with human IgG control protein. Mice were treated with 200µg/dose/mouse of sIL-13R α 2.Fc on days -3, -2, -1 prior to the outcome day in each protocol, with a further 200µg dose given 1 hour before outcome measurements (**Figure 1**). In the brief model, this treatment was given on days 18, 19, 20 and 21 (i.e. day before and 2 days of challenge, and then day of outcome). In the chronic model treatment was given in the last week of the 4-week recovery period after the final allergen challenge on day 90. The following outcome measurements were made; 1) *in vivo* airway responsiveness to intravenous methacholine; 2) total and differential cell counts in bronchoalveolar lavage (BAL) fluid; and 3) airway morphometry using a computer based image analysis system. Separate groups of 10 mice were studied in each treatment arm of each protocol.

Airway Responsiveness:

The underlying contributors to airway responsiveness, namely airway reactivity (slope of increase in total respiratory system resistance (R_{RS}) for a given increase in methacholine (MCh) dose), airway sensitivity (lowest dose of MCh to produce bronchoconstriction) and the maximum inducible bronchoconstriction (maximum R_{RS}) were measured following brief or chronic allergen challenge (**Figure 2**). These components of airway responsiveness were measured based on the response of R_{RS} to saline and increasing intravenous doses of MCh (**Figure 2**). R_{RS} was measured using the flow interrupter technique, modified for use in mice, and described in detail elsewhere ^{34,37,39}.

Bronchoalveolar Lavage (BAL): Following airway physiology measurements, BAL was performed as described by us previously ³⁷. Differential cell counts were performed on 400 cells, counted by one investigator blind to the experimental conditions. IL-13 protein levels in BAL fluid were assessed by enzyme-linked immunosorbant assay (Quantikine, R&D Systems, Minneapolis, MN). Levels below 7.8 pg/mL are considered by the manufacturer to be below the level of detection.

Lung Histology and Morphometry: The lungs were dissected and processed as described by us in detail previously ³⁴. Three- μ m thick transverse sections were cut and assessed with the following stains: hematoxylin and eosin (H&E) for the presence of eosinophils; picrosirius red (PSR) to demonstrate the presence of collagen, and periodic acid Schiff (PAS) to demonstrate the presence of mucin within goblet cells. Additional sections were prepared for immunohistochemistry using a monoclonal antibody (clone α sm-1, Novacastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom) against α -smooth muscle actin (α -SMA) to identify contractile elements. (We use the term 'contractile elements' rather than contractile smooth muscle, as this immuno-stain identifies the alpha smooth muscle actin contractile protein present in both contractile and secretory smooth muscle phenotypes and in myofibroblasts). Morphometric quantification of the stained lung sections was performed using a customized digital image analysis system (Northern Eclipse, Empix Imaging Inc., Mississauga, Canada), as described by us in detail previously ^{34,40}.

Statistical Analysis:

Reported values are expressed as mean and standard error of the mean (SEM). Comparisons with respect to airway reactivity (slope of the R_{RS} - log transformed MCh dose response curve), maximal bronchoconstriction (maximal MCh induced R_{RS}), cell counts and indices of airway remodeling between saline control mice and mice receiving either brief or chronic allergen exposure, treated with either sIL-13R α 2.Fc or IgG control protein, were made using analysis of variance (ANOVA). Post-hoc multiple comparison testing was performed using Duncan's test to assess for significant effects. All comparisons were two-tailed, and p values < 0.05 were considered to be significant.

Results

1. Treatment with sIL-13Rα2.Fc during brief allergen exposure prevents the development of Th2 mediated AHR.

Following a period of brief allergen challenge, mice treated with IgG control protein exhibited a significant increase in total cell counts and eosinophils in BAL when compared to mice exposed to saline and treated with IgG control protein (p<0.001) (**Figure 3**). BAL IL-13 levels increased from 71.7 (SEM 2.4) pg/ml to 122.9 (SEM 13.6) pg/ml in saline vs. allergen challenged mice (p<0.05). Treatment with sIL-13R α 2.Fc during the period of brief allergen challenge (**Figure 1**) resulted in significant attenuation of the allergen-induced increase in total cell counts (p=0.02) but had no significant affect on the number of eosinophils in BAL fluid (**Figure 3**). The magnitude of BAL eosinophilia in response to brief allergen challenge, and the subsequent lack of attenuation by sIL-13R α 2.Fc treatment was similar to that seen in the airway tissue (data not shown).

Following brief exposure to allergen, mice treated with IgG control protein exhibited significant increases in airway reactivity (p<0.001) and maximal bronchoconstriction (p<0.001), compared to the saline control groups (**Figure 4**). We were also able to confirm, as others have shown before ^{1,2}, that sIL-13R α 2.Fc treatment during the period of brief allergen challenge completely abrogated the development of airway hyperreactivity and the increased maximal bronchoconstriction present in allergen challenged / IgG control treated mice (**Figure 4**).

2. Chronic allergen exposure results in aspects of airway wall remodeling and sustained *AHR* that is not attenuated by sIL-13Rα2.Fc treatment.

As in the brief challenge protocol, sIL-13R α 2.Fc or IgG control treatment was given during the 4 days prior to outcome measurements being made, but the timing of this intervention in the chronic protocol meant that treatment with sIL-13R α 2.Fc was only given some 3 weeks after the final allergen challenge (**Figure1**), at a time when airway remodeling was established ³⁴. BAL IL-13 measured at this time was not different between saline challenged (67.0; SEM 6.8 pg/ml) and allergen challenged (66.6; SEM 5.2 pg/ml) mice (p>0.05). Following chronic allergen exposure, IgG control protein treated mice exhibited a significant and sustained increase in airway reactivity (p<0.01) and maximal bronchoconstriction (p<0.01) (**Figure 5**), compared to saline control mice. In contrast to our observations in the brief exposure model, treatment with sIL-13R α 2.Fc after chronic allergen exposure had no attenuating effect on either this sustained airway hyperreactivity or the sustained increase in maximal bronchoconstriction (**Figure 5**). There was a significant increase in the amount of mucin-containing PAS positive goblet cells (Figures 6, 7A and 7B), subepithelial collagen deposition (Figures 6, 7D and 7E) and α -SMA staining (Figures 6, 7G and 7H) in the airways of the IgG control treated mice 4 weeks after chronic allergen exposure, compared to saline control mice (p<0.01). As we had anticipated *a priori*, neutralization of IL-13 had no effect on any of these indices of airway remodeling (Figures 6, 7C, 7F and 7I). Consistent with our previous studies ³⁴, minimal numbers of eosinophils were observed in the BAL fluid of IgG control treated and sIL-13R α 2.Fc treated mice 4 weeks after chronic allergen exposure, and these values were not significantly different to baseline values observed in saline control mice (Figure 3).

Discussion

This is the first study to directly examine the role of IL-13 in a model of established, sustained AHR. Our main purpose with this study was to demonstrate an anti-IL-13 insensitive component of allergen induced AHR. We therefore waited until a time point following chronic allergen challenge when IL-13 levels had decreased, and blocked any residual biological activity of IL-13 using the sIL-13R α 2.Fc fusion protein. The results from our study clearly illustrate that blocking IL-13 activity at this time is not capable of reversing the sustained AHR

Although there have been other reports of animal models in which chronic allergen or fungal exposure has resulted in structural airway changes and AHR⁴¹⁻⁴⁵, our model is fundamentally different in that the sustained AHR we observe is present for at least 8 weeks after the final allergen challenge, at a time when acute Th2-mediated inflammatory responses have largely resolved ³⁴. This is in contrast to the AHR described in the other models of chronic allergen or fungal exposure, where AHR is observed at times when cellular airway inflammation is still
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marked ^{41,43-45}. We have now shown that neutralizing IL-13, at a time when chronic allergen exposure has already resulted in the establishment of airway remodeling, did not attenuate the sustained AHR observed in our model. These observations are consistent with our underlying hypothesis, namely that IL-13 mediated effector pathways are not required to maintain sustained AHR; they also provide further evidence that sustained AHR is a consequence of airway remodeling, rather than ongoing Th2 cytokine-mediated airway inflammation. We do however recognize that earlier Th2 immune-mediated inflammatory events are likely to be critical in the initial pathogenesis of functionally important airway remodeling processes, as evidenced by the fact that IL-13 gene-deficient mice are protected from developing aspects of airway remodeling or sustained AHR when subjected to this chronic allergen exposure protocol ³⁵.

We have also shown that specific neutralization of IL-13 in mice during a period of brief allergen exposure, at a time when IL-13 protein levels were significantly increased in BAL fluid compare to saline control mice, prevented the development of transient, Th2-cytokine mediated AHR. By demonstrating that treatment with sIL-13R α 2.Fc was able to completely prevent this component of AHR following brief allergen challenge, we have confirmed that sIL-13R α 2.Fc is functionally effective at neutralizing the effects of endogenous IL-13. Our observations thus confirm the critical role of IL-13 in initiating the acute Th2-mediated inflammatory events that lead to transient AHR in this model and are consistent with other published reports in which IL-13 blockade resulted in the abrogation of allergen-induced AHR^{1,2}. However, while these experimental models of brief allergen exposure point to the fact that IL-13 may be an important therapeutic target in the treatment of AHR, they only allow evaluation of interventions on acute Th2-mediated responses, and do not take into account the chronic structural changes that are characteristic of established asthma and which have been implicated in the pathophysiology of AHR.

In agreement with previous observations by us, BAL IL-13 levels were not elevated above control levels in chronically challenged mice ³⁵. We felt however, that this was not sufficient for concluding that anti-IL-13 treatment would not be effective at this time. Ongoing local production of IL-13 may well have been affecting smooth muscle function in these mice without elevating BAL IL-13 levels. Here however, we have shown that anti-IL-13 treatment at this time was ineffective, indicating that ongoing production of IL-13 was not playing a role in maintaining AHR.

We recognize that immunological tolerance is likely to occur during our chronic challenge protocol. This may decrease the contribution of IL-13 to the sustained AHR present in our chronic model. Nonetheless, the major focus of the present study was to demonstrate that there is an IL-13 independent component of AHR; this aspect of AHR should not be affected by the development of immunological tolerance. We would interpret the results of our present study as demonstrating that, while both tolerance and the blocking of IL-13 reduce the immune-mediated component of AHR, there is a substantial residual component of AHR that is *independent* of immune-mediated mechanisms and the effects of IL-13. This interpretation would be wholly consistent with our overall hypothesis that there is an IL-13 independent component of AHR

Given that transgenic over-expression of IL-13 results in tissue eosinophilia in mouse airways ³, we might have expected that sIL-13R α 2.Fc treatment would result in a more substantial attenuation of the allergen-induced airway eosinophilia than was noted in this study. However, our results are also consistent with a previously published report ¹ in that IL-13 blockade prior to brief allergen exposure did not significantly attenuate allergen-induced airway

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eosinophilia. It is also consistent with our own previous work in which IL-13 gene deficient mice had only a modest reduction in tissue eosinophilia compared to wild-type control mice, following brief periods of allergen exposure ³⁵. These observations suggest that eosinophil biology is likely influenced by a number of mediators and cytokines, in addition to IL-13. It is also likely that IL-13 dependent AHR occurs by mechanisms that are independent of airway eosinophilia.

In this study we have not attempted to reverse structural changes, but rather to leave them intact and remove a specific immune mediator. For this reason we elected to treat mice with sIL-13R α 2.Fc for a relatively brief 4-day period. While this was sufficient to neutralize IL-13 in the airways and to address our study hypothesis, we recognized *a priori* that this treatment regimen was likely to be too brief to have any effect on the indices of airway remodeling measured in the study. Thus, our observations that IL-13 blockade had no attenuating effect on indices of remodeling were entirely expected; they do however point to the IL-13 independent contribution that chronic structural changes play in the pathogenesis of sustained AHR.

Our study design also provides an opportunity to speculate on the potential therapeutic effects of extended sIL-13R α 2.Fc treatment during the period of chronic allergen exposure. Interventions that regulate Th2 cytokine effector pathways are attractive as potential therapeutic targets, and we assume that concomitant sIL-13R α 2.Fc treatment throughout the period of chronic allergen exposure might result in abrogation of Th2 immune-mediated airway inflammation, with the subsequent attenuation of aspects of airway remodeling and AHR. It is however perhaps more intriguing to speculate on whether prolonged sIL-13R α 2.Fc treatment, given after a period of chronic allergen exposure, might facilitate some resolution of the AHR and associated airway remodeling. Data from pre-clinical studies suggest a potential impact of IL-13 antagonism on airway remodeling, with evidence that sIL-13R α 2.Fc treatment

significantly blocks collagen formation in a model of hepatic fibrosis ⁴⁶, and also prevents collagen deposition in chronic allergic airway inflammation ⁴⁷. Formal testing of this hypothesis has important implications for targeted anti-IL-13 as a potential therapy for asthma, and is clinically relevant in that the majority of patients with asthma already have aspects of airway remodeling present at the time of clinical presentation ⁴⁸.

In summary, we have demonstrated that neutralization of IL-13 during a period of brief allergen challenge, at a time when acute Th2-mediated airway inflammation is associated with transient AHR, results in the prevention of this component of AHR. In contrast, neutralization of IL-13 after chronic repeated allergen exposure, at a time when airway remodeling has already become established, has no effect on the sustained AHR. Our results strongly support the paradigm that the transient airway hyperreactivity occurring after brief exposure to allergen is dependent on IL-13 mediated effector pathways. However, our observations extend that understanding by providing substantial novel evidence that there is a component of AHR that is associated with aspects of airway remodeling, and that blocking the effect of biologically active IL-13 has no effect on this component of AHR. Our observations also imply that anti-IL-13 strategies may be no more effective in the clinical management of established asthma than currently available inhaled corticosteroid therapies that target immune-mediated inflammation ⁴⁹.

References

- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. Science 1998; 282: 2258-2261.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998; 282: 2261-2263.
- Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999; 103: 779-788.
- Yang M, Hogan SP, Henry PJ, Matthaei KI, McKenzie AN, Young IG, Rothenberg ME, Foster PS. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. Am J Respir Cell Mol Biol 2001; 25: 522-530.
- Mattes J, Yang M, Siqueira A, Clark K, MacKenzie J, McKenzie AN, Webb DC, Matthaei KI, Foster PS. IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. J Immunol 2001; 167: 1683-1692.
- Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, Hogan SP, Koskinen A, McKenzie AN, Dent LA, Rothenberg ME, Matthaei KI, Young IG, Foster PS. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin

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precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. J Exp Med 2002; 195: 1433-1444.

- Taube C, Duez C, Cui ZH, Takeda K, Rha YH, Park JW, Balhorn A, Donaldson DD, Dakhama A, Gelfand EW. The role of IL-13 in established allergic airway disease. J Immunol 2002; 169: 6482-6489.
- 8. Morse B, Sypek JP, Donaldson DD, Haley KJ, Lilly CM. Effects of IL-13 on airway responses in the guinea pig. Am J Physiol Lung Cell Mol Physiol 2002; 282: L44-L49.
- Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med 2002; 8: 885-889.
- Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. J Clin Invest 2003; 111: 291-297.
- Wills-Karp M, Chiaramonte M. Interleukin-13 in asthma. Curr Opin Pulm Med 2003; 9: 21-27.
- Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. Chemokines in asthma: cooperative interaction between chemokines and IL-13. J Allergy Clin Immunol 2003; 111: 227-242.
- 13. Wynn TA. IL-13 effector functions. Annu Rev Immunol 2003; 21: 425-456.
- 14. O'Byrne PM, Inman MD. Airway hyperresponsiveness. Chest 2003; 123: 411S-416S.

- 15. Jeffery PK, Godfrey E, Adelroth E, Nelson F, Rogers A, Johansson S-A. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. Am Rev Respir Dis 1992; 145: 890-899.
- 16. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am J Respir Crit Care Med 1999; 159: 1043-1051.
- 17. Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, Chakir J. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence Of inhaled corticosteroids. Am J Respir Crit Care Med 2000; 162: 1308-1313.
- Leigh R, Vethanayagam D, Yoshida M, Watson RM, Rerecich T, Inman MD, O'Byrne PM. Effects of montelukast and budesonide on airway responses and airway inflammation in asthma. Am J Respir Crit Care Med 2002; 166: 1212-1217.
- James AL, Pare PD, Hogg JC. The mechanics of airway narrowing in asthma. Am Rev Respir Dis 1989; 139: 242-246.
- Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989; 140: 1745-1753.

- Wiggs BR, Bosken C, Pare PD, James A, Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1992; 145: 1251-1258.
- Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. J Appl Physiol 1993; 74: 2771-2781.
- Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1993; 148: 1220-1225.
- Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, Boutet M. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. Chest 1997; 112: 45-52.
- Boulet LP, Chakir J, Dube J, Laprise C, Boutet M, Laviolette M. Airway inflammation and structural changes in airway hyper- responsiveness and asthma: an overview. Can Respir J 1998; 5: 16-21.
- 26. Laprise C, Laviolette M, Boutet M, Boulet LP. Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling. Eur Respir J 1999; 14: 63-73.
- Wang L, McParland BE, Pare PD. The functional consequences of structural changes in the airways: implications for airway hyperresponsiveness in asthma. Chest 2003; 123: 356S-362S.

- 28. Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1989; 1: 520-524.
- 29. Carroll N, Elliot J, Morton A, James A. The structure of large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis 1993; 147: 405-410.
- Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. J Clin Invest 1999;
 104: 1001-1006.
- Busse W, Elias J, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. Am J Respir Crit Care Med 1999; 160: 1035-1042.
- Bousquet J, Jeffery PK, Busse WB, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. Am J Respir Crit Care Med 2000; 161: 1720-1745.
- Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001; 164: S28-S38.
- 34. Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergeninduced airway inflammation. Am J Respir Cell Mol Biol 2002; 27: 526-535.
- 35. Leigh R, Ellis R, Wattie JN, Hirota JA, Matthaei KI, Foster PS, O'Byrne PM, Inman MD. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. Am J Respir Crit Care Med 2004; 169: 860-867.

- Leigh, R., Ellis, R., Wattie, J., Donaldson, D. D., and Inman, M. D. Interleukin-13 is not required to maintain airway dysfunction in mice following chronic allergen exposure. [abstract]. Am J Respir Crit Care Med 2004; 169: A576.
- Inman MD, Ellis R, Wattie J, Denburg JA, O'Byrne PM. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. Am J Respir Cell Mol Biol 1999; 21: 473-479.
- 38. Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, O'Hara RM, Jr., Beier DR, Turner KJ, Wood CR, Collins M. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. J Immunol 1998; 161: 2317-2324.
- 39. Volgyesi GA, Tremblay LN, Webster P, Zamel N, Slutsky AS. A new ventilator for monitoring lung mechanics in small animals. J Appl Physiol 2000; 89: 413-421.
- 40. Ellis R, Leigh R, Southam DS, O'Byrne PM, Inman MD. Morphometric Analysis of Mouse Airways Following Chronic Allergen Challenge. Lab Invest 2003; 83: 1285-1291.
- 41. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax 1998; 53: 849-856.
- 42. Trifilieff A, El Hashim A, Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. Am J Physiol Lung Cell Mol Physiol 2000; 279: L1120-L1128.

- 43. Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 2000; 161: 627-635.
- 44. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL, Lukacs NW. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. Am J Pathol 2000; 156: 723-732.
- 45. Tanaka H, Masuda T, Tokuoka S, Takahashi Y, Komai M, Nagao K, Nagai H. Time course study on the development of allergen-induced airway remodeling in mice: the effect of allergen avoidance on established airway remodeling. Inflamm Res 2002; 51: 307-316.
- 46. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J Clin Invest 1999; 104: 777-785.
- Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, Hogaboam CM. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. J Immunol 2001; 166: 5219-5224.
- 48. Busse WW, Lemanske RF, Jr. Asthma. N Engl J Med 2001; 344: 350-362.
- Inman M. Do complex conditions require complex treatment? Am J Respir Crit Care Med 2003; 167: 6.

Figure Legends

Figure 1: Study protocols.

Sensitization and challenge protocols used in brief and chronic challenge models. Note that sIL- $13R\alpha 2.Fc / IgG$ control treatment was given 4 days prior to outcome measurements being made in each protocol.

Figure 2: Airway responsiveness methods.

Total respiratory system resistance (R_{RS}) was measured in response to increasing doses of i.v. MCh. Using the resulting R_{RS} -MCh dose response curve, indices of airway reactivity (Slope R_{RS}), airway sensitivity, or the lowest dose to produce bronchoconstriction (Break R_{RS}) and maximal degree of bronchoconstriction (Max R_{RS}) were measured.

Figure 3: BAL cell counts following brief or chronic exposure to saline or allergen.

Total cell counts (TCC) and eosinophil counts measured in BAL fluid, following brief challenge with either saline (SAL) or ovalbumin (OVA) and treatment with either IgG control antibody (DIL) or sIL-13R α 2.Fc (anti-IL-13).

Figure 4: Airway responses following brief exposure to saline or allergen.

Maximum airway bronchoconstriction and airway reactivity, calculated as the dose response slope to intravenous MCh, measured 24h following brief exposure to saline or allergen.

Figure 5: Airway responses following chronic exposure to saline or allergen.

Maximum airway bronchoconstriction and airway reactivity, calculated as the dose response slope to intravenous MCh, measured 4 weeks following brief exposure to saline or allergen.

Figure 6: Morphometric changes in airways of mice following chronic exposure to saline or allergen.

Morphometric quantification of mucin containing goblet cells (PAS), collagen (PSR), and contractile elements (α -SMA) in the airways of mice following chronic challenge with saline (SAL) or ovalbumin (OVA) and treatment with IgG control antibody (DIL) or sIL-13R α 2.Fc (anti-IL-13). Data are expressed as the number of goblet cells staining for mucin (PAS positive), expressed per length (cells/mm) of airway wall, and as the percentage of positively stained tissue in the region of interest in the PSR and α -SMA stained sections.

* indicates p < 0.05 compared to saline challenged mice.

Figure 7: Histological sections of airway wall from chronically challenged mice.

Staining for PAS positive goblet cells (**Panels A-C**), collagen deposition (PSR, viewed using polarized light microscopy) (**Panels D-F**), and contractile elements (α SMA) (**Panels G-I**) in the airways of mice following chronic exposure to saline with IgG control antibody treatment (**Panels A, D, G**), allergen with IgG control antibody treatment (**Panels B, E, H**), or allergen with sIL-13R α 2.Fc treatment (**Panels C, F, I**). Bars indicate 50-µm.

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anti-IL-13



Chapter 8

DISCUSSION

AHR has long been recognized as a characteristic feature of asthma. It is therefore not surprising that much of the research exploring the pathogenesis of asthma has focused on the mechanisms underlying AHR. Over the past two decades, a number of experimental and clinical allergen-challenge models have been used to investigate the mechanisms of AHR. While this line of research has told us much about the immunemediated molecular and signaling mechanisms involved in the pathogenesis of AHR, it has also facilitated a somewhat one-sided, and perhaps simplistic view of a phenomenon that, in reality, is complex and likely to be multi-factorial. It has also meant that airway remodeling has received considerably less attention as a potential contributor to the pathogenesis of AHR. Yet chronic structural changes of the airway, involving epithelial cell and goblet cell hyperplasia and metaplasia, collagen deposition and thickening of the subepithelial region, smooth muscle hypertrophy and hyperplasia, and proliferation of airway blood vessels and nerves are a consistent histological feature in almost all patients with established asthma (1-5). Furthermore, mathematical modeling studies have indicated that these chronic structural changes are theoretically able to account for a large component of AHR (6-10). Thus, while it appears that characteristic inflammatory processes of asthma play a role in determining AHR, it is increasingly evident that additional factors are also involved in the pathogenesis of AHR. This concept is further supported by the fact that, although allergen challenge or steroid treatment are capable of either markedly increasing or attenuating the degree of eosinophilic airway inflammation

respectively (11-15), such interventions are associated with relatively small changes in AHR, with improvements in PC_{20} methacholine (or histamine) of only 1-2 doubling concentrations. Furthermore, in studies where the extent of eosinophilic inflammation has been related to the degree of AHR, such relationships are usually weak and indeed sometimes absent (5;16-18). Taken together, these observations have given rise to the hypothesis that AHR may result from two temporally independent processes, namely, a variable responsive component, which can be attributed to fluctuating degrees of immune-mediated airway inflammation, and a sustained component, that remains relatively fixed and which is relatively independent of acute inflammatory events within the airway. This hypothesis also suggests that airway remodeling may be functionally relevant in the pathogenesis of AHR, and may provide an explanation for the sustained, corticosteroid-resistant AHR (19), the accelerated decline in lung function observed over time in patients with established asthma (20;21), and the loss of corticosteroid responsiveness on baseline lung function observed in the Childhood Asthma Management Program study (22). The overall purpose of the studies contained in this thesis has been to explore the mechanisms underlying AHR, and to segregate the contributions of immunemediated inflammatory responses and chronic structural changes to the physiological consequences of chronic allergen inhalation.

In the first study contained in this thesis, (Chapter 2), we examined the effects of inhaled budesonide and montelukast, alone or in combination, on outcome variables after allergen inhalation in 10 subjects with mild asthma. Although a number of studies have confirmed that regular inhaled corticosteroid therapy inhibits the late asthmatic response,

it does not significantly attenuate the early asthmatic response and results in only a modest attenuation of allergen-induced AHR (12;13). Furthermore, inhaled steroid treatment does not completely abrogate allergen-induced increases in sputum eosinophils (13;14;23). We therefore reasoned that there might be other ongoing inflammatory mechanisms, which are relatively insensitive to the anti-inflammatory effects of inhaled corticosteroid therapy that might contribute to the pathogenesis of AHR. Cysteinyl leukotrienes are inflammatory mediators that cause potent bronchocontriction and are thought to be critical in the pathogenesis of the early asthmatic response (24-26). However, their inflammatory effects may not be inhibited by corticosteroid treatment (27), and we hypothesized that the combination of an inhaled corticosteroid (budesonide) and a leukotriene receptor antagonist (montelukast) would provide greater antiinflammatory effects than either drug alone. We further postulated that this greater abrogation of allergen-induced airway inflammation may result in greater protection against the allergen induced AHR. However, while treatment with either drug, alone or in combination, resulted in significant attenuation of allergen-induced sputum eosinophilia and afforded protection of approximately 1-doubling concentration against allergeninduced AHR, combination treatment did not provide greater protection against allergeninduced AHR than did either drug alone. Also, combination treatment did not result in the normalization of airway responses in the study subjects. This study was novel in that it was the first published report to directly compare the protection afforded by an inhaled corticosteroid with that afforded by montelukast, either alone or in combination, on allergen-induced airway inflammation and AHR. The results indicated that, while

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combination therapy was effective in attenuating airway eosinophilia as well as the early and late asthmatic responses, this was associated with only a modest 1-2 fold doubling concentration protection against allergen-induced AHR, and was no more effective in this regard than treatment with either drug alone. The results of this study also confirmed that it is unlikely that acute inflammatory events in the airway account for all of aspects of AHR, and further supported our hypothesis that other factors, including airway remodeling, may be important in the pathogenesis of sustained AHR. The remaining papers contained in this thesis describe, firstly, the development of a novel mouse model in which chronic exposure to allergen results in *sustained* airway dysfunction and some of the features of airway remodeling that are representative of asthma, and secondly, explore some of the mechanisms underlying the physiological consequences of chronic allergen exposure.

In Chapter 3, we describe, in detail, the development of a chronic allergen challenge model in mice that results in the development of sustained airway dysfunction, which is associated with structural changes in the airway consistent with aspects of airway remodeling seen in asthma. In this study we observed that mice subjected to a single period of allergen exposure developed *transient* airway hyperreactivity that was associated with increases in airway eosinophilia and bronchoalveolar lavage (BAL) IL-13 levels. In these mice, airway inflammation and airway reactivity returned to normal over the next 2-4 weeks. In contrast, following chronic exposure to allergen, mice developed *sustained* airway hyperreactivity that persisted for at least 8 weeks after the final period of allergen exposure. This was well beyond the point at which both the eosinophilia and IL-

13 levels had returned to baseline levels. Histological examination of airway tissue was performed using morphometric image analysis techniques (described in detail in **Chapter** 4). Subepithelial fibrosis of the airways was not observed at any time following brief allergen exposure, but was evident at 4 and 8 weeks following chronic allergen exposure. There were increases in mucin staining at 24 hours and 2 weeks following the final allergen challenge in both models, but this only persisted beyond that time in the chronic model. Finally, increased contractile staining was observed in both groups of mice at all time points after challenge. Consistent with modeling studies of the airway (8;10), this increased contractile tissue was associated with greater maximal degrees of bronchoconstriction, which persisted throughout the 8-week period following both brief and chronic allergen exposure.

In addition to our own laboratory, several other laboratories have also recently developed models of chronic exposure to allergen or fungus in order to observe the effects on airway wall remodeling and AHR (28-40). These models are consistent in that they all result in AHR and various indices of airway remodeling, including sub-epithelial fibrosis, goblet and epithelial cell hyperplasia, and increased amounts of contractile tissue. As a group, these studies also support the concept that chronic allergic type inflammation is sufficient to produce many of the chronic pathologic lesions associated with the asthmatic airway. However, our model is fundamentally different, in that we have demonstrated, for the first time, the development of *sustained* airway hyperreactivity following chronic exposure to allergen that was not associated with the presence of Th2 mediated airway inflammation, such as airway eosinophilia or IL-13. Furthermore, we

observed indices of airway remodeling that were unique to chronically challenged mice, and which appeared to contribute to the presence of *sustained* airway hyperreactivity. Thus, we have developed a novel mouse model of chronic airway allergic inflammation in which chronic allergen exposure results in two of the key features of asthma; 1/ a fluctuating component of AHR, temporally linked to acute inflammatory events in the airway, and 2/ a sustained component of AHR that is independent of these events, but appears to be linked to structural remodeling of the airway, similar to that seen in asthma. Our results from this study indicate that *transient* airway dysfunction following brief exposure to allergen, and *sustained* airway dysfunction following chronic allergen exposure, appear to be two interrelated but distinct entities.

In **Chapter 4** we have validated the morphometric technique used to quantify indices of airway remodeling in all the mouse model experiments described in this thesis. In this study, we demonstrated that the technique provides an objective valid, and reliable method to assess structural changes in the airways of mice, following chronic allergen exposure. The technique allows investigators to objectively quantify morphologic changes in mouse airways, thereby minimizing investigator bias associated with many of the more subjective techniques currently in use. We also provided sample size estimates for future studies using this chronic allergen exposure model, by demonstrating that the power to detect structural remodeling changes is influenced by the depth beneath the epithelium that is analyzed by the morphometric image analysis technique. This is the first time that such a validation study of the technique has been reported, and provides a reference standard for the methodology of the technique, as well as providing useful information regarding the numbers of animals required for future studies.

The Th2 cytokines IL-4, IL-5 and IL-13 have each been identified as mediators of allergic airway inflammation, and it has been thought that they indirectly facilitate the development of AHR by promoting the recruitment and subsequent activation of eosinophils and other inflammatory cells into the airway (41-47). It has been postulated that these inflammatory cells then act on resident airway cells to initiate the chronic structural changes that result in AHR. Alternatively, it has been suggested that these Th2 cytokines act directly on resident airway cells to promote aspects of airway remodeling and subsequent AHR (48-53). In Chapter 5 we report a series of experiments in which we sought to determine whether the presence of IL-4, IL-5 or IL-13 was necessary for the development of indices of airway remodeling or the development of sustained airway hyperreactivity. In this study, both IL-4 and IL-13 gene deficient mice were completely protected from developing aspects of airway remodeling and sustained airway hyperreactivity following chronic allergen exposure. In contrast, and despite the virtual absence of tissue eosinophilia, IL-5 gene deficient mice developed aspects of airway remodeling and sustained airway hyperreactivity, similar to that seen in wild type mice following chronic allergen exposure. These results strongly support the concept that airway remodeling and sustained airway dysfunction occur as a consequence of repeated Th2 immune-mediated airway inflammation, and they illustrate that IL-4 and IL-13, but not IL-5, are critical for the development of these phenomena. They also provide substantial novel evidence that some of the remodeling changes observed in the mouse airways following chronic allergen inhalation are likely to play an important role in the pathophysiology of *sustained* airway hyperreactivity.

The findings in this study are largely consistent with the published literature, which has suggested that IL-4 is required for the development of AHR (54), while IL-5, although necessary for the development of eosinophilic airway inflammation (42:54-56) is not required for the development of AHR (45;54;55;57;58). Similarly, a number of reports have consistently shown that IL-13 is required for the development of AHR (47;59-61). However, ours is the first study to examine the requirement for these cytokines in the pathogenesis of sustained AHR. These results are also consistent with a previous clinical study of mepolizumab (humanized anti-IL-5 blocking monoclonal antibody) treatment in asthma, which reported no effect on baseline or allergen-induced AHR, despite a substantial reduction in blood and sputum eosinophilia at the highest dose used (18). Thus, despite their apparently close association, airway eosinophilia would seem not to be a critical requirement for the phenotypic expression of AHR. However, the methodology in this clinical study has been questioned, in that it was statistically underpowered, while subjects in the placebo-control arm did not demonstrate allergeninduced AHR in the absence of treatment (62). More recently, Flood-Page and colleagues (63) have reported that treatment of subjects with mild asthma with mepolizumab reduced circulating, sputum and tissue eosinophil numbers (by approximately 90%, 60-80%, and 55% from baseline respectively), but had no significant effect on AHR. In a further publication using the same cohort of study subjects, the authors report that mepolizumab treatment reduced the deposition of some, but not all extracellular matrix proteins in the

subepithelial basement membrane of the airways, compared to placebo treatment (64). Taken together, these findings demonstrate that anti-IL-5 blocking mAb treatment fails to deplete eosinophils from the airway, and that even attenuating, rather than depleting, airway eosinophils and eosinophil degranulation results in some reduction of matrix protein deposition in the airways. Thus, we should be cautious in excluding the eosinophil as a potential mechanism in the pathogenesis of airway remodeling and AHR. We also need to be mindful of interpreting our own study results in realizing that we cannot be certain that eosinophils are likely to be functionally different from human eosinophils (65). Clearly, further studies are needed to determine whether eosinophils contribute to airway remodeling processes and the development of sustained AHR in human asthma.

Results from the preceding studies indicate that airway remodeling processes and sustained airway dysfunction are likely to develop secondarily to repeated Th2 type inflammatory events; they also indicate that sustained airway dysfunction persists *beyond* the resolution of these immune-mediated inflammatory responses. We therefore wondered whether blocking specific Th2 immune-mediated inflammatory responses, at a time when airway remodeling was established, would be sufficient to attenuate this sustained airway dysfunction. In **Chapter 6** and **Chapter 7** we examined the effects of immuno-depleting CD4⁺ and CD8⁺ T cells (**Chapter 6**) or blocking the effects of IL-13 (**Chapter 7**), either at the time of brief allergen exposure when Th2 immune-mediated mechanisms were likely to account for transient airway hyperreactivity, or at a later time-point when indices of airway remodeling were established, and sustained airway

hyperreactivity was present. The major findings from these series of experiments were firstly, that the airway dysfunction associated with ongoing Th2 responses could be abrogated by preventing mediator release from T-cells, or by blocking IL-13, and secondly that these immuno-depletion interventions were not effective at reversing *sustained* airway dysfunction that was established several weeks after a period of chronic allergen challenge (at a time when acute Th2-mediated inflammation has largely resolved). The results of these two studies are novel, in that they point to a complex basis for AHR in diseases such as asthma, and they also have potentially important clinical implications, in suggesting that immune based therapies, or therapies aimed at reducing ongoing inflammation, may never be capable of reversing airway responsiveness to normal levels in patients with established asthma. Rather, in order to achieve this ultimate goal, either immuno-modulatory intervention will need to be initiated earlier in the course of asthma pathogenesis, or additional therapies or environments will need to be developed that allow restoration of a normal (or at least functionally normal) airway wall in patients with established asthma.

It is apparent from the studies contained in this thesis, as well as from the many other published studies in the field, that AHR may result from a number of underlying mechanisms. The results from our studies support the current paradigm that considers AHR to be one of the major consequences of airway inflammation and remodeling. However, while AHR is generally seen in the setting of acute immune-mediated airway inflammation, the relationship between acute inflammatory processes and the chronic structural changes of airway remodeling in the pathophysiology of AHR has not yet been

well characterized (66). It is however increasingly recognized that structural remodeling processes within the airway occur early during the development of asthma, and have been demonstrated to be present in bronchial biopsy specimens from young children taken several years before asthma became clinically evident (67). Therefore, part of the difficulty in evaluating the specific roles for these mechanisms is that many of the fundamental scientific questions are difficult, if not impossible, to answer in human studies. As a result, we, along with many other investigators, have elected to utilize appropriate animal models to characterize the potential mechanisms that underlie the functional consequences of allergic airway disease.

Our findings from the studies presented in this thesis offer an advance in the level of our understanding of the immuno-pathological mechanisms of AHR that was previously provided by murine models based on brief allergen exposure protocols (58-60). These studies have focused on understanding the mechanisms of acute, immunemediate airway inflammation and the associated transient airway dysfunction that occur following a single, brief period of exposure to allergen. This line of research has told us much about the functionally important molecular and signaling mechanisms involved in the transient airway dysfunction present in these models. For example, IL-13 appears to play a key role in AHR, while IL-5 plays a key role in eosinophilic inflammation without necessarily having a functional consequence. Data from these studies propose that there are two distinct ways through which inflammation can promote airway hyperreactivity. Firstly, inflammatory cells (mast cells or perhaps inflammatory cells present in the submucosa but not in the airways) are able to release histamine, leukotrienes, platelet activating factor and proteases, that have relatively short half-lives and promote acute bronchoconstriction and increased AHR. Secondly, eosinophils can release MBP and other granule proteins, which activate macrophages to release cytokines and chemokines that may act on airway structural cells to promote airway remodeling. It is not necessarily clear whether this line of research targeting acute, immune-mediated inflammatory mechanisms, will result in therapies that are superior to inhaled corticosteroids. Indeed, inhaled corticosteroids have already been shown to have outstanding anti-inflammatory effects, and are highly effective at attenuating the inflammation and airway dysfunction associated with brief allergen exposure.

Our studies provide supportive evidence that the airway remodeling and sustained airway dysfunction observed following chronic allergen challenge is as a result of repeated Th2-mediated inflammatory processes in the airway. We have observed that various interruptions of these Th2 inflammatory processes during the time of brief allergen exposure results in substantial attenuation of the *transient*, inflammationassociated AHR. In addition to our own chronic allergen model, a number of other investigators have developed murine models in which chronic exposure to allergen results in several indices of airway remodeling, and in some cases, sustained AHR (30-32;34-36;39;40;68-70). While these and accumulating results will help to tell us much about the mechanisms of sustained dysfunction in asthma, it is not yet clear how they will lead to improved pharmacology available for the asthmatic patient. Nonetheless, with increased understanding of the relationship between airway remodeling and sustained AHR provided by these models, the aim should be to develop treatment strategies that will address the component of AHR, which is not affected by currently available therapy.

It has already been established that topical corticosteroids in rats (70) and systemic anti-leukotrienes in mice (34) are capable of attenuating indices of airway remodeling when treatment was applied throughout periods of chronic allergen challenge. In the case of corticosteroid treatment, this effect was associated with prevention of sustained AHR (70). However, in the case of anti-leukotriene treatment AHR persisted, which may have been a result of ongoing acute immune events as measurements were made shortly after cessation of allergen exposure (34). It is important to emphasize that in the Vanacker study (70), topical corticosteroids were only effective at preventing indices of airway remodeling and sustained AHR when delivered throughout the period of allergen exposure; initiation of therapy *after* allergen exposure was ineffective in terms of attenuating airway pathology and function. These findings are perhaps not surprising, given our current understanding that a large component of AHR in asthma appears to be steroid insensitive. However, what these findings do suggest is that the extent and functional consequences of airway remodeling may be moderated if anti-inflammatory therapy with inhaled steroids and possibly other agents, including anti-leukotrienes, is initiated very early in the course of the disease. Whether such an approach is clinically feasible, given that the structural changes of airway remodeling are already present in early childhood and may precede the onset of asthma symptoms (67), remains to be established. The more common and challenging clinical scenario is that of patients who present with symptoms of asthma, at a time when the structural changes of airway remodeling are already established (71-73). In this context, studies have established that

treatment, including topical steroids, anti-leukotrienes and various molecular interventions, are able to attenuate airway remodeling to some degree, but to date no interventions have been able to demonstrate a functionally important reversal of this process (34;64;70). Our chronic model is therefore relevant in that it provides a novel opportunity to further our understanding of the non-acute inflammatory contributors to AHR, and may be useful in developing new treatment strategies that will address that those components of AHR that seem to be resistant to anti-inflammatory treatment.

There are a number of outstanding research questions, relating specifically to our model, that remain to be answered, and which are likely to provide further novel insights into the pathophysiology of AHR. The first question relates to the fact that AHR appears to have a strong genetic component; genetic studies in human asthma point to different patterns of inheritance for AHR (74-76), and it has been proposed that a genetic predisposition to develop abnormal repair processes within the airway, secondary to various stimuli or inflammatory insults, likely plays an important role in predisposing individuals to asthma (77-79) This paradigm is further supported by the recent identification of a novel asthma susceptibility gene, ADAM33, which is a member of the ADAM (a disintegrin and metalloprotease) family of zinc-dependent metalloproteases that are encoded on chromosome 20p13 (80). In linkage analyses, statistical significance was strengthened when AHR was included in the asthma phenotype but weakened when asthma was conditioned for serum IgE or allergen-specific IgE levels, indicting a gene more closely linked to altered airway function than to allergic inflammation per se (80).

At present, it is not apparent which tissue(s) contribute the most to the development of AHR. However, a recent report has described substantial phenotypic differences in the degree of airway dysfunction and structural remodeling changes in the airways of different mouse strains subjected to the same chronic allergen challenge protocol (81). A similar study, currently underway in our laboratory, will evaluate differences in the degree of sustained airway hypereactivity and indices of airway remodeling in different strains of mice, and will likely provide important clues as to which components of airway remodeling are more relevant regarding in the development of AHR. It is perhaps more tantalizing to hypothesize as to whether different indices of remodeling are influenced by different genetic control mechanisms; if this is the case, then specific gene deletion studies will facilitate quantification of the contribution of each of the structural components of airway remodeling to the pathophysiology of AHR.

A second direction that needs to be further explored is the potential role of other indices of airway remodeling in the development of AHR. Both neuronal and vascular (enlargement of existing vessels as well as neo-vascularization) changes have been observed in the airways of asthmatic individuals (82-87). To date, we have not looked for evidence of either vascular or neuronal remodeling in our chronic model. It will be interesting to quantify whether such changes exist, and if so, whether they have any relationship to the sustained airway hyperreactivity that is present in the model. Identification of additional indices of airway remodeling, particularly if they are strongly associated with airway dysfunction, will provide additional potential therapeutic targets in the treatment of AHR.

A third question that remains to be answered is whether currently available therapies, including topical corticosteroids, anti-leukotrienes and various molecular interventions, are able to effect functionally important attenuations of any of the indices of airway remodeling, if treatment is initiated *after* the establishment of airway remodeling but then given for a prolonged period of time. We believe this is analogous to clinical practice, where patients usually present after the disease process is already established. Results from such a study will address the hypothesis of whether the early introduction of anti-inflammatory therapy is useful in reducing the development of airway remodeling and sustained AHR.

In conclusion, it is likely that a number of different mechanisms interact to produce AHR. While we have proposed that there may be separate mechanisms responsible for the underlying AHR in asthma, and for the variability seen throughout the course of the disease, it is possible that this distinction is not complete. It is likely that immune-mediated inflammatory mechanisms, together with chronic structural changes, in combination with airway wall edema, combine to cause airway wall thickening and AHR. It is also likely that the underlying mechanisms responsible for the immune-mediated inflammatory cell recruitment and mediator release may, in the short-term, be responsible for the variability in AHR, as well as, in the longer-term, for the underlying structural changes that are responsible for *sustained* AHR. Increased airway responsiveness to a variety of non-specific physical, chemical or pharmacological bronchoconstrictor agonists may possibly develop when airway inflammation and remodeling have progressively increased over months or years, and symptomatic asthma could represent the final
outcome of such a process. Nonetheless, the results from the studies contained in this thesis support the paradigm that different mechanisms are involved in causing different components of AHR. It is likely that one mechanism (airway remodeling) is responsible for the underlying AHR in asthmatic patients, differentiating them from healthy individuals, while another mechanism (acute, immune-mediated airway inflammation) is important for the changes in AHR seen within individual asthmatic subjects during the course of the disease.

<u>References</u>

- 1. Dunnill MS, Massarella GR, and Anderson JA: A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. Thorax 1969, 24: 176-179
- 2. Sobonya RE: Quantitative structural alterations in long-standing allergic asthma. Am Rev Respir Dis 1984, 130: 289-292
- 3. Saetta M, di Stefano A, Rosina C, Thiene G, and Fabbri LM: Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. Am Rev Respir Dis 1991, 143: 138-143
- 4. Carroll N, Elliot J, Morton A, and James A: The structure of large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis 1993, 147: 405-410
- 5. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, and Kay AB: Bronchial biopsies in asthma:An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989, 140: 1745-1753
- 6. James AL, Pare PD, and Hogg JC: The mechanics of airway narrowing in asthma. Am Rev Respir Dis 1989, 139: 242-246
- 7. Wiggs BR, Moreno R, Hogg JC, Hilliam C, and Pare PD: A model of the mechanics of airway narrowing. J Appl Physiol 1990, 69: 849-860
- 8. Wiggs BR, Bosken C, Pare PD, James A, and Hogg JC: A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1992, 145: 1251-1258
- 9. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, and Hogg JC: Small airways dimensions in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1993, 148: 1220-1225
- Lambert RK, Wiggs BR, Kuwano K, Hogg JC, and Pare PD: Functional significance of increased airway smooth muscle in asthma and COPD. J Appl Physiol 1993, 74: 2771-2781
- 11. Jeffery PK, Godfrey E, Adelroth E, Nelson F, Rogers A, and Johansson S-A: Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. Am Rev Respir Dis 1992, 145: 890-899
- 12. Gauvreau GM, Doctor J, Watson RM, Jordana M, and O'Byrne PM: Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. Am J Respir Crit Care Med 1996, 154: 1267-1271

- 13. Gauvreau GM, Wood LJ, Sehmi R, Watson RM, Dorman SC, Schleimer RP, Denburg JA, and O'Byrne PM: The effects of inhaled budesonide on circulating eosinophil progenitors and their expression of cytokines after allergen challenge in subjects with atopic asthma. Am J Respir Crit Care Med 2000, 162: 2139-2144
- 14. Inman MD, Watson RM, Rerecich T, Gauvreau GM, Lutsky BN, Stryszak P, and O'Byrne PM: Dose-dependent effects of inhaled mometasone furoate on airway function and inflammation after allergen inhalation challenge. Am J Respir Crit Care Med 2001, 164: 569-574
- 15. Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, and Chakir J: Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence Of inhaled corticosteroids. Am J Respir Crit Care Med 2000, 162: 1308-1313
- 16. Gibson PG, Dolovich J, Denburg JA, Ramsdale EH, and Hargreave FE: Chronic cough: eosinophilic bronchitis without asthma. Lancet 1989, 1: 1346-1348
- 17. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, and Brusasco V: Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. Am J Respir Crit Care Med 1998, 157: 4-9
- 18. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, and Barnes PJ: Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000, 356: 2144-2148
- 19. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, and Sterk PJ: Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am J Respir Crit Care Med 1999, 159: 1043-1051
- 20. Lange P, Parner J, Vestbo J, Schnohr P, and Jensen G: A 15-year follow-up study of ventilatory function in adults with asthma. N Engl J Med 1998, 339: 1194-1200
- 21. Rasmussen F, Taylor DR, Flannery EM, Cowan JO, Greene JM, Herbison GP, and Sears MR: Risk factors for airway remodeling in asthma manifested by a low postbronchodilator FEV1/vital capacity ratio: a longitudinal population study from childhood to adulthood. Am J Respir Crit Care Med 2002, 165: 1480-1488

- Long-term effects of budesonide or nedocromil in children with asthma. The Childhood Asthma Management Program Research Group. N Engl J Med 2000, 343: 1054-1063
- 23. Wood LJ, Sehmi R, Gauvreau GM, Watson RM, Foley R, Denburg JA, and O'Byrne PM: An inhaled corticosteroid, budesonide, reduces baseline but not allergen-induced increases in bone marrow inflammatory cell progenitors in asthmatic subjects. Am J Respir Crit Care Med 1999, 159: 1457-1463
- 24. Dahlen S-E, Hedqvist P, Hammarstrom S, and Samuelsson B: Leukotrienes are potent constrictors of human bronchi. Nature 1980, 286: 484-486
- 25. Lewis RA, Austen KF, and Soberman RJ: Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. N Engl J Med 1990, 323: 645-655
- 26. Drazen JM, Israel E, and O'Byrne PM: Treatment of Asthma with Drugs Modifying the Leukotriene Pathway. N Engl J Med 1999, 340: 197-206
- 27. Dworski R, Fitzgerald GA, Oates JA, and Sheller JR: Effect of oral prednisone on airway inflammatory mediators in atopic asthma. Am J Respir Crit Care Med 1994, 149: 953-959
- Blease K, Jakubzick C, Schuh JM, Joshi BH, Puri RK, and Hogaboam CM: IL-13 fusion cytotoxin ameliorates chronic fungal-induced allergic airway disease in mice. J Immunol 2001, 167: 6583-6592
- 29. Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, and Hogaboam CM: Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. J Immunol 2001, 166: 5219-5224
- 30. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, and Kumar RK: An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax 1998, 53: 849-856
- 31. Trifilieff A, El Hashim A, and Bertrand C: Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. Am J Physiol Lung Cell Mol Physiol 2000, 279: L1120-L1128
- 32. Blyth DI, Wharton TF, Pedrick MS, Savage TJ, and Sanjar S: Airway subepithelial fibrosis in a murine model of atopic asthma: suppression by dexamethasone or anti-interleukin-5 antibody. Am J Respir Cell Mol Biol 2000, 23: 241-246

- 33. Foster PS, Ming Y, Matthei KI, Young IG, Temelkovski J, and Kumar RK: Dissociation of inflammatory and epithelial responses in a murine model of chronic asthma. Lab Invest 2000, 80: 655-662
- 34. Henderson WR, Jr., Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, and Chi EY: A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. Am J Respir Crit Care Med 2002, 165: 108-116
- 35. Kenyon NJ, Ward RW, and Last JA: Airway fibrosis in a mouse model of airway inflammation. Toxicol Appl Pharmacol 2003, 186: 90-100
- 36. Masuda T, Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwara D, and Nagai H: Mast cells play a partial role in allergen-induced subepithelial fibrosis in a murine model of allergic asthma. Clin Exp Allergy 2003, 33: 705-713
- 37. Moir LM, Leung SY, Eynott PR, McVicker CG, Ward JP, Chung KF, and Hirst SJ: Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. Am J Physiol Lung Cell Mol Physiol 2003, 284: L148-L159
- 38. Salmon M, Walsh DA, Koto H, Barnes PJ, and Chung KF: Repeated allergen exposure of sensitized Brown-Norway rats induces airway cell DNA synthesis and remodelling. Eur Respir J 1999, 14: 633-641
- 39. Tanaka H, Masuda T, Tokuoka S, Takahashi Y, Komai M, Nagao K, and Nagai H: Time course study on the development of allergen-induced airway remodeling in mice: the effect of allergen avoidance on established airway remodeling. Inflamm Res 2002, 51: 307-316
- 40. Palmans E, Kips JC, and Pauwels RA: Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 2000, 161: 627-635
- 41. Gavett SH, Chen X, Finkelman F, and Wills-Karp M: Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. Am J Respir Cell Mol Biol 1994, 10: 587-593
- 42. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, and Young IG: Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 1996, 183: 195-201

- 43. Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, and Locksley RM: Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. Molecular Medicine 1998, 4: 344-355
- 44. Shi HZ, Xiao CQ, Zhong D, Qin SM, Liu Y, Liang GR, Xu H, Chen YQ, Long XM, and Xie ZF: Effect of inhaled interleukin-5 on airway hyperreactivity and eosinophilia in asthmatics. Am J Respir Crit Care Med 1998, 157: 204-209
- 45. Hogan SP, Matthaei KI, Young JM, Koskinen A, Young IG, and Foster PS: A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. J Immunol 1998, 161: 1501-1509
- 46. Shi HZ, Deng JM, Xu H, Nong ZX, Xiao CQ, Liu ZM, Qin SM, Jiang HX, Liu GN, and Chen YQ: Effect of inhaled interleukin-4 on airway hyperreactivity in asthmatics. Am J Respir Crit Care Med 1998, 157: 1818-1821
- 47. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, and Elias JA: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999, 103: 779-788
- 48. Postlethwaite AE, Holness MA, Katai H, and Raghow R: Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. J Clin Invest 1992, 90: 1479-1485
- 49. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Canonica GW, Jasmin C, and Azzarone B: Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. J Clin Invest 1998, 101: 2129-2139
- 50. Richter A, Puddicombe SM, Lordan JL, Bucchieri F, Wilson SJ, Djukanovic R, Dent G, Holgate ST, and Davies DE: The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. Am J Respir Cell Mol Biol 2001, 25: 385-391
- 51. Ihn H, Yamane K, Asano Y, Kubo M, and Tamaki K: IL-4 Up-Regulates the Expression of Tissue Inhibitor of Metalloproteinase-2 in Dermal Fibroblasts Via the p38 Mitogen-Activated Protein Kinase-Dependent Pathway. J Immunol 2002, 168: 1895-1902
- 52. Mullings RE, Wilson SJ, Puddicombe SM, Lordan JL, Bucchieri F, Djukanovic R, Howarth PH, Harper S, Holgate ST, and Davies DE: Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium. J Allergy Clin Immunol 2001, 108: 832-838

- 53. Lordan JL, Bucchieri F, Richter A, Konstantinidis A, Holloway JW, Thornber M, Puddicombe SM, Buchanan D, Wilson SJ, Djukanovic R, Holgate ST, and Davies DE: Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. J Immunol 2002, 169: 407-414
- 54. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, and Locksley RM: Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J Exp Med 1996, 183: 109-117
- 55. Hessel EM, Van Oosterhout AJ, Van A, I, Van Esch B, Hofman G, Van Loveren H, Savelkoul HF, and Nijkamp FP: Development of airway hyperresponsiveness is dependent on interferon- gamma and independent of eosinophil infiltration. Am J Respir Cell Mol Biol 1997, 16: 325-334
- 56. Wang J, Palmer K, Lotvall J, Milan S, Lei XF, Matthaei KI, Gauldie J, Inman MD, Jordana M, and Xing Z: Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia. J Clin Invest 1998, 102: 1132-1141
- 57. Tanaka H, Nagai H, and Maeda Y: Effect of anti-IL-4 and anti-IL-5 antibodies on allergic airway hyperresponsiveness in mice. Life Sci 1998, 62: L169-L174
- 58. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, and Foster PS: Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. J Immunol 2000, 165: 108-113
- 59. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, and Donaldson DD: Interleukin-13: central mediator of allergic asthma. Science 1998, 282: 2258-2261
- 60. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, and Corry DB: Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998, 282: 2261-2263
- 61. Zhu Z, Lee CG, Zheng T, Chupp G, Wang J, Homer RJ, Noble PW, Hamid Q, and Elias JA: Airway inflammation and remodeling in asthma. Lessons from interleukin 11 and interleukin 13 transgenic mice. Am J Respir Crit Care Med 2001, 164: S67-S70
- O'Byrne PM, Inman MD, and Parameswaran K: The trials and tribulations of IL-5, eosinophils, and allergic asthma. J Allergy Clin Immunol 2001, 108: 503-508

- 63. Flood-Page PT, Menzies-Gow AN, Kay AB, and Robinson DS: Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. Am J Respir Crit Care Med 2003, 167: 199-204
- 64. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, and Kay AB: Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. J Clin Invest 2003, 112: 1029-1036
- 65. Persson CG: Con: mice are not a good model of human airway disease. Am J Respir Crit Care Med 2002, 166: 6-7
- 66. Holgate ST: Asthma: more than an inflammatory disease. Curr Opin Allergy Clin Immunol 2002, 2: 27-29
- 67. Pohunek, P., Roche, W. R., Tarzikova, J., Kurdmann, J., and Warner, J. O. Eosinophilic inflammation in the bronchial mucosa in children with bronchial asthma. Eur Respir J 11[suppl 25]. 2000.
- 68. Kips JC, Cuvelier CA, and Pauwels RA: Effect of acute and chronic antigen inhalation on airway morphology and responsiveness in actively sensitized rats. Am Rev Respir Dis 1992, 145: 1306-1310
- 69. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL, and Lukacs NW: Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. Am J Pathol 2000, 156: 723-732
- 70. Vanacker NJ, Palmans E, Kips JC, and Pauwels RA: Fluticasone inhibits but does not reverse allergen-induced structural airway changes. Am J Respir Crit Care Med 2001, 163: 674-679
- 71. Laitinen LA, Laitinen A, Altraja A, Virtanen I, Kampe M, Simonsson BG, Karlsson SE, Hakansson L, Venge P, and Sillastu H: Bronchial biopsy findings in intermittent or "early" asthma. J Allergy Clin Immunol 1996, 98: S3-S6
- 72. Laitinen A, Karjalainen EM, Altraja A, and Laitinen LA: Histopathologic features of early and progressive asthma. J Allergy Clin Immunol 2000, 105: S509-S513
- 73. Busse WW and Lemanske RF, Jr.: Asthma. N Engl J Med 2001, 344: 350-362
- 74. Skadhauge LR, Christensen K, Kyvik KO, and Sigsgaard T: Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. Eur Respir J 1999, 13: 8-14

- 75. Leaves NI, Bhattacharyya S, Wiltshire S, and Cookson WO: A detailed genetic map of the chromosome 7 bronchial hyper-responsiveness locus. Eur J Hum Genet 2002, 10: 177-182
- 76. Allen M, Heinzmann A, Noguchi E, Abecasis G, Broxholme J, Ponting CP, Bhattacharyya S, Tinsley J, Zhang Y, Holt R, Jones EY, Lench N, Carey A, Jones H, Dickens NJ, Dimon C, Nicholls R, Baker C, Xue L, Townsend E, Kabesch M, Weiland SK, Carr D, von Mutius E, Adcock IM, Barnes PJ, Lathrop GM, Edwards M, Moffatt MF, and Cookson WO: Positional cloning of a novel gene influencing asthma from chromosome 2q14. Nat Genet 2003, 35: 258-263
- 77. Boulet LP, Chakir J, Dube J, Laprise C, Boutet M, and Laviolette M: Airway inflammation and structural changes in airway hyper- responsiveness and asthma: an overview. Can Respir J 1998, 5: 16-21
- 78. Holgate ST: Genetic and environmental interaction in allergy and asthma. J Allergy Clin Immunol 1999, 104: 1139-1146
- 79. Davies DE, Wicks J, Powell RM, Puddicombe SM, and Holgate ST: Airway remodeling in asthma: new insights. J Allergy Clin Immunol 2003, 111: 215-225
- 80. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, Walsh A, Liu Z, Hayward B, Folz C, Manning SP, Bawa A, Saracino L, Thackston M, Benchekroun Y, Capparell N, Wang M, Adair R, Feng Y, Dubois J, FitzGerald MG, Huang H, Gibson R, Allen KM, Pedan A, Danzig MR, Umland SP, Egan RW, Cuss FM, Rorke S, Clough JB, Holloway JW, Holgate ST, and Keith TP: Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature 2002, 418: 426-430
- 81. Shinagawa K and Kojima M: Mouse model of airway remodeling: strain differences. Am J Respir Crit Care Med 2003, 168: 959-967
- Saetta M and Turato G: Airway pathology in asthma. Eur Respir J Suppl 2001, 34: 18s-23s
- 83. Goldie RG, Rigby PJ, Fernandes LB, and Henry PJ: The impact of inflammation on bronchial neuronal networks. Pulm Pharmacol Ther 2001, 14: 177-182
- 84. Piedimonte G: Contribution of neuroimmune mechanisms to airway inflammation and remodeling during and after respiratory syncytial virus infection. Pediatr Infect Dis J 2003, 22: S66-S74

- 85. McDonald DM: Angiogenesis and remodeling of airway vasculature in chronic inflammation. Am J Respir Crit Care Med 2001, 164: S39-S45
- 86. Chetta A, Zanini A, Foresi A, Del Donno M, Castagnaro A, D'Ippolito R, Baraldo S, Testi R, Saetta M, and Olivieri D: Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. Am J Respir Crit Care Med 2003, 167: 751-757
- 87. Tanaka H, Yamada G, Saikai T, Hashimoto M, Tanaka S, Suzuki K, Fujii M, Takahashi H, and Abe S: Increased airway vascularity in newly diagnosed asthma using a high-magnification bronchovideoscope. Am J Respir Crit Care Med 2003, 168: 1495-1499

APPENDIX

Methods Used in Mouse Model Experiments

While all the manuscripts that make up this 'sandwich" thesis provide a description of the methods used in each study, the following paragraphs provide a more detailed description of several of the methods used in the mouse model experiments. Some of the methods, such as airway morphometry, have been described in detail in several of the publications. However, because many journals place limitations on the space allowed for the description of methods, some of the methods in our manuscripts have been described in less detail; many times the reader is referred to previous publications for a more detailed understanding of the rationale and principles of the methods used. These additional descriptions of the methods used in our mouse model experiments should allow the reader to fully understand the principles underlying several of the methods used in our studies, without having to refer back to previous publications.

Sensitization and Challenge: Mice are sensitized with intraperitoneal ovalbumin (IP OVA) injections on days 1 and 11, and intranasal ovalbumin (IN OVA) on day 11. Briefly, IP OVA is prepared by precipitating 10% aluminum potassium sulphate with 0.05% ovalbumin (Grade V, Sigma Chemical Co, St Louis, MO) in sterile Dulbecco's phosphate buffered saline (PBS); the pH of the resultant suspension is adjusted to 6.5 by adding 2.5N NaOH. The suspension is then centrifuged, washed 3 times to remove excess aluminum, and the pellet re-suspended in sterile PBS; 200-µl is then injected into awake mice intraperitoneally on days 1 and 11. Sham sensitized mice are injected with saline precipitated in alum. Intranasal ovalbumin (IN OVA), administered on day 11 and subsequently as part of the challenge protocols (see individual study protocols), involves dissolving 4 mg ovalbumin in 1 ml sterile PBS; $25-\mu l$ (100- μg OVA) PBS is then instilled intra-nasally into mice lightly anesthetized with isoflurane (Abbott Laboratories, Ltd. Montreal, PQ Canada) according to the study protocol. This is best accomplished by using a P200 Gilson pipette to slowly eject the fluid onto the tip of the nasal cavity, and then allowing it to enter passively with respiration.

Airway Responsiveness: in our laboratory, we measure airway responsiveness to increasing intravenous doses of methacholine (MCh) on the basis of the response of total respiratory system resistance (R_{RS}) (1). In this model, R_{RS} is measured using the flow interrupter technique, as modified for use with mice (2). Briefly, mice are anesthetized using 2,2,2-tribromoethanol (Avertin, 240mg/kg intraperitoneally; Aldrich Chemical CO., Milwaukee, WI), and when anesthesia is established, the trachea is exposed and cannulated using a blunted 18-gauge needle. The needle is then attached to a ventilator (RV5; Voltek Enterprises, Inc., Toronto, ON, Canada) designed to deliver constant inspiratory flow and tidal volume delivery independent of respiratory parameter changes that occur during the MCh challenge. The initial pattern of ventilation is based on a tidal volume of 0.1 ml/kg delivered over 45 ms, with a 530-ms end-inspiratory pause and a 95-ms period of passive expiration (breathing frequency of 95 breaths/min). Heart rate and oxygen saturation are 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. After the mouse has been stabilized on the ventilator, the internal jugular vein is cannulated using a 25-gauge needle. Paralysis is achieved using pancuronium bromide (0.03 mg/kg intravenously; Sabex Inc, Boucherville, PQ Canada) to prevent respiratory effort during measurement.

The response of R_{RS} is then measured after intravenous injections of saline, and then 10, 33, 100 and 330 mg/kg of MCh (ACIC, Brantford, ON Canada), each delivered as a 0.2-ml bolus dose. To establish a constant volume history, mice are subjected to three inspirations to total lung capacity (TLC) (end-inspiratory pressure of 30 cm H2O) followed by 60 seconds of 90-breaths per minute ventilation before each dose. Upon injection, the ventilatory pattern is changed so that the time allowed for passive expiration is extended to 1,425-ms thus reducing the breathing frequency to 30 breaths per minute as suggested by Volgyesi and associates (3). This change is done to prevent the dynamic hyperinflation (also termed breath-stacking) that is observed during MCh challenge when mice are ventilated with shorter expiratory times. Following the peak in R_{RS} (20 to 30 s) the breathing pattern is returned to 90 breaths per minute. When R_{RS} has returned to baseline, the mouse is again inflated three times to TLC and ventilated for 90 seconds before beginning the next dose. During each MCh dosing, the mouth-pressure signal from the ventilator is converted to a digital signal (Dash 16, Metrabyte, Staughton, MA) and recorded at 400 Hz on a PC computer. R_{RS} and respiratory system elastance are calculated as described previously (2). Briefly, compliance is measured based on the pressure and volume differences between the periods of no flow before and after the inspiration. R_{RS} is calculated by subtracting the compliance-associated pressure from the signal during inspiration and relating it to the flow rate at a simultaneous point. R_{RS} and compliance are thus measured for the respiratory system. The measurements are made at a single time point in inspiration only. Evaluation of airway responsiveness is based on the peak R_{RS} measured in the 30 seconds after the saline and Mch challenges. An index of airway reactivity is calculated as the slope of the straight-line regression between peak R_{RS} and the log₁₀ of the MCh dose, using the data from the 10, 33, and 100 µg/kg doses. The data at the 330-µg/kg dose is not included in this regression because peak R_{RS} has frequently reached a plateau at this dose. Furthermore, we have also previously observed a marked reduction in heart rate at this dose (1). An index of airway sensitivity is calculated as the MCh dose at which the above regression intersects with the baseline R_{RS} (peak R_{RS} after the saline challenge). The maximum inducible bronchoconstriction was measured as the greatest R_{RS} achieved with any MCh dose.

Bronchoalveolar Lavage (BAL): Following airway physiology measurements, and under continued anesthesia (Avertin, Aldrich Chem., Milwaukee, Wis, 240mg/kg i.p.), the chest cavity is opened and the mouse is sacrificed by performing cardiac puncture and terminal exsanguination. This procedure is performed prior to BAL, so as to prevent extravasation of blood into BAL fluid. Two aliquots of 250-µl phosphate buffered saline are then injected and withdrawn through the blunted 18-gauge needle previously used to cannulate the trachea. The BAL fluid is centrifuged for 10 min at 150 g and 21°C. The supernatant is stored at -20 °C for future analyses.The cell pellet is then resuspended in PBS and a total cell count performed using a hemocytometer. The cells

are then diluted to an approximate concentration of 5 x 10⁵/ml with PBS, cytocentrifuge slides are prepared (Cytospin 3; Shandon Scientific, Sewickley, PA) and stained with Diff-Quik® Fixative (Dade Behring Inc., Newark, DE). Differential cell counts are performed on 400 cells, by one investigator blind to the experimental conditions. Cells are classified, based on morphological criteria, as macrophages, neutrophils, lymphocytes or eosinophils.

References

- 1. Inman MD, Ellis R, Wattie J, Denburg JA, and O'Byrne PM: Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. Am J Respir Cell Mol Biol 1999, 21: 473-479
- Levitt RC, Eleff SM, Zhang LY, Kleeberger SR, and Ewart SL: Linkage homology for bronchial hyperresponsiveness between DNA markers on human chromosome 5q31-q33 and mouse chromosome 13. Clin Exp Allergy 1995, 25 Suppl 2: 61-63
- 3. Volgyesi GA, Tremblay LN, Webster P, Zamel N, and Slutsky AS: A new ventilator for monitoring lung mechanics in small animals. J Appl Physiol 2000, 89: 413-421