

IMPACT OF CHRONIC AIRWAY INFLAMMATION ON ASTHMA
PATHOGENESIS

IMPACT OF CHRONIC ALLERGIC INFLAMMATION ON *DE NOVO*
SENSITIZATION AND AIRWAY REMODELING IN A
MOUSE MODEL OF ALLERGIC AIRWAY DISEASE

By

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— ABSTRACT —

Allergic asthma is a chronic inflammatory disease of the airways. Importantly, the chronic nature of this disease imparts specific additional consequences that would not otherwise be observed in a strictly acute setting. The development of various structural alterations to the airway wall, collectively termed airway remodeling, represents one such example. Decades of research have provided a great deal of insight into the acute allergic asthmatic response and the processes that govern it. However, less is known about the impact of protracted allergen exposure and chronic immune-inflammatory responses. To this end, the research presented in this thesis explores the consequences of chronic allergen exposure and persistent airway inflammation on asthma pathogenesis, using a mouse model of allergic airway disease induced by respiratory exposure to house dust mite (HDM) allergens. Specifically examined are: i) the impact of continuous allergen exposure and the resulting immune-inflammatory response on the development of *de novo* sensitization to newly encountered allergens (Chapter 2) and, ii) the roles of transforming growth factor (TGF)- β and eosinophils, two putatively critical components of the allergic inflammatory response, in the generation of airway remodeling (Chapters 3 and 4). Our data show that chronic exposure to HDM facilitates the development of the full ‘asthmatic phenotype’ towards an innocuous antigen. Moreover, they demonstrate that, unlike what has been previously observed in ovalbumin-based models, neither TGF- β nor eosinophils are critically required for remodeling to develop in the context of HDM exposure. These findings highlight the importance of the lung microenvironment in influencing the type of immune response that develops upon initial antigen encounter and, furthermore, underscore the notion that the role of a particular cell type or molecule in the asthmatic response is contextual and not necessarily broadly applicable.

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— LIST OF ABBREVIATIONS —

Ab	antibody	IL	interleukin
Ag	antigen	KLH	keyhole limpet hemocyanin
AHR	airway hyperresponsiveness	LPS	lipopolysaccharide
APC	antigen presenting cell	MBP	major basic protein
BAL	bronchoalveolar lavage	MMP	matrix metalloproteinase
CEA	common environmental allergen	OVA	ovalbumin
CCL	CC-chemokine ligand	PDGF	platelet derived growth factor
CTGF	connective tissue growth factor	RSV	respiratory syncytial virus
DC	dendritic cell	SMC	smooth muscle cell
DEP	diesel exhaust particle	T β R I	TGF- β receptor type-I
ECM	extracellular matrix	T β R II	TGF- β receptor type-II
EGF	epidermal growth factor	TGF- β	transforming growth factor- β
EPO	eosinophil peroxidase	Th2	type-2 T cells
Fc ϵ RI	high affinity IgE receptor	TIMP	tissue inhibitor of metalloproteinase
FGF	fibroblast growth factor	TLR	toll-like receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor	TSLP	thymic stromal lymphopoietin
HDM	house dust mite	VEGF	vascular endothelial growth factor
Ig	immunoglobulin	WT	wild-type

— PREFACE —

The research documented in Chapters 2-4 of this doctoral thesis represent three independent although conceptually related bodies of work that, as of July 2009, have been previously published or are in the submission process. The work conducted in each manuscript required a collaborative effort with several colleagues resulting in multiple authors.

CHAPTER 2 Fattouh R, Pouladi MA, Alvarez D, Johnson JR, Walker TD, Goncharova S, Inman MD, Jordana M. House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am J Respir Crit Care Med*. 2005 Aug 1;172(3):314–21. Epub 2005 May 5.

This study was conducted during June 2002 – December 2004. As the lead author I designed and performed all of the experiments, analyzed all data and wrote the manuscript. M.A. Pouladi (graduate student), D. Alvarez (graduate student), J.R. Johnson (graduate student), TD Walker (technologist) and S. Goncharova (technologist) provided me with experimental assistance. Dr. M.D. Inman was responsible for the measurement of airway physiology and provided valuable scientific input.

CHAPTER 3 Fattouh R, Midence NG, Arias K, Johnson JR, Walker TD, Goncharova S, Souza KP, Gregory RC Jr, Lonning S, Gauldie J, Jordana M. Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *Am J Respir Crit Care Med*. 2008 Mar 15;177(6):593–603. Epub 2008 Jan 3.

This study was completed between May 2004 – October 2007. As the primary author I designed and performed all of the experiments, analyzed all data and wrote the manuscript.

N.G. Midence was an undergraduate student who worked on this project under my supervision. K. Arias (graduate student), J.R. Johnson, T.D. Walker, and S. Goncharova provided technical assistance with the experiments. K.P. Souza (Genzyme collaborator) and R.C. Gregory (Genzyme collaborator) provided scientific advice and assisted with the measurements of TGF- β and anti-TGF- β antibody. S. Lonning (Genzyme collaborator) provided us with the anti-TGF- β antibody and critically appraised the manuscript. J. Gauldie provided expert scientific input throughout the study and also critically appraised the manuscript.

CHAPTER 4 Fattouh R, Al-Garawi A, Fattouh M, Arias K, Walker TD, Goncharova S, Coyle AJ, Humbles AA, Jordana M. Eosinophils are Dispensable for House Dust Mite-Induced Allergic Airway Remodeling and Immunity. Submitted to *Am J Respir Crit Care Med*. May 2009.

This study was conducted between the period of September 2007 – August 2009. As the principal author I designed and performed all of the experiments, analyzed all data and wrote the manuscript. Experimental assistance was provided by A. Al-Garawi (graduate student), K. Arias, T.D. Walker and S. Goncharova. M. Fattouh (undergraduate student) assisted with morphometric analysis. A.J. Coyle (MedImmune collaborator) and A.A. Humbles (MedImmune collaborator) provided us with the Δ dbl GATA line of eosinophil-deficient mice, contributed valuable scientific input and critically appraised the manuscript.

— Chapter 1 —

INTRODUCTION

The past several decades have witnessed an astounding increase in our knowledge of the mechanisms that underlie the pathogenesis of allergic asthma. Yet, this has been paralleled by an equally dramatic increase in the prevalence of this disease (1, 2). This has been especially true of so-called ‘westernized’ regions such as North America, the United Kingdom, and Western Europe (1, 3, 4) although, more recently, this trend has been observed in less-developed countries as well (1, 3). It is estimated that, in westernized countries up to 40% of the population is sensitized to common environmental allergens (CEAs) including tree and grass pollens, as well as dust mite and animal materials (5). The International Study of Asthma and Allergy in Children reported that in countries with the highest prevalence of allergic disease, approximately 25% of children aged 13-14 years display ‘symptoms’ of allergic asthma (i.e. wheezing) (1, 6). In Canada, survey data in which the diagnosis of asthma was made by a health professional indicate that the prevalence of asthma among children, 0-11 years old, increased to more than 13% in 2000/01, up from 11% in 1994/95, and to 8.4% in those 12 years or older, up from <4% in the early 1980’s (7, 8). Globally, it is estimated that up to 300 million people are affected (3). Asthma affects people of all ages and races, slightly more boys than girls (ages 0-11) but more women than men and provokes symptoms that are constant sources of distress interfering with sleep, recreational activities, and intellectual function and, in some cases, are life threatening (7-9). Encouragingly, prevalence rates seem to have been stabilizing over the past 5-10 years and data from the 1990’s onward note lower proportions of high-severity symptoms and fewer asthma-related hospitalizations (i.e. asthma attacks) and fatalities (7, 8, 10, 11). Nonetheless, if we are to maintain and improve

upon these favourable trends the need for diligent research into the origins and pathogenesis of allergic asthma is unequivocal.

Allergic Asthma and the Allergic Immune-Inflammatory Response

Allergic asthma is a chronic immune-inflammatory driven disease that is characterized clinically by reversible airflow obstruction and airway hyperresponsiveness (AHR) and is associated with the development of various physical alterations to the structure of the airway walls. Conceptually, the type of immune response that is typically thought to predominate in allergic asthma is a specialized form that has been termed the ‘type-2’ or ‘Th2’ response, named after the cell type generally considered to be the central orchestrator of allergic immune responses: the ‘type-2’ T-helper cell (12, 13). Although the allergic response involves an array of cells and molecules (immune and otherwise), hallmark factors traditionally include Th2 cells, B cells, eosinophils, mast cells, the interleukins (IL)-4, -5, -9 and -13 and immunoglobulins (Ig)E and IgG₁ (12, 13). Ultimately, a variety of immune phenotypes make up the response and, for this reason, it is perhaps more appropriately referred to as Th2-polarized. That the fundamental goal of immunity is to protect the host against harmful infectious agents makes it difficult to comprehend why such immune responses are generated against seemingly harmless antigens (Ags), i.e. allergens, in the first place. Given that the majority of the world’s population is free from such disease suggests that tolerance¹ rather than inflammation is the natural response to aeroallergens.

¹ A state of specific and intended immunological hyporesponsiveness

From an immune perspective asthma can be crudely separated into two phases: i) sensitization and ii) elicitation (refer to Figure 1); although in reality both processes occur, to some extent, as a continuum. Sensitization, in this case, refers to the immunological process whereby an individual acquires the ability to mount an Ag-specific allergic immune response i.e. activates adaptive immunity and acquires Th2-polarized immunological memory to a given allergen. The prevailing notion is that, upon allergen

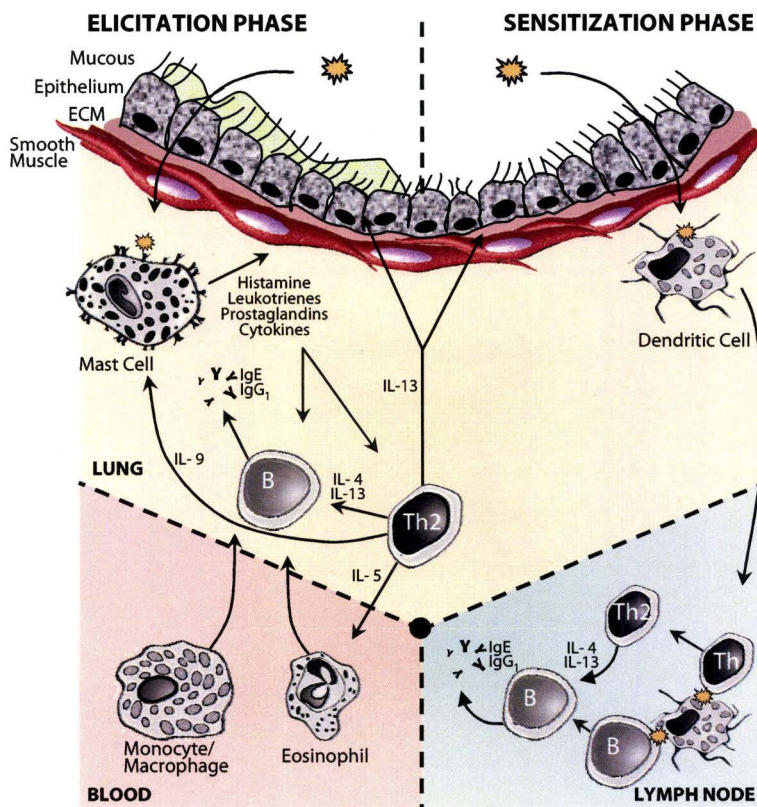


Figure 1. Simplified schematic of i) allergic airway sensitization and ii) the subsequent elicitation of allergic airway responses. Sensitization Phase – Upon initial encounter, dendritic cells phagocytose the allergen, migrate to draining lymph nodes and trigger Th2 cell differentiation and proliferation along with B cell activation. Th2 cells stimulate allergen-specific B cells to undergo Ig isotype switching (IgE and IgG₁). Elicitation phase – Upon subsequent encounter, IgE cross-linking on the surface of mast cells induces the production of various mediators that stimulate smooth muscle causing bronchoconstriction. Mediator release also recruits Th2 cells, among other inflammatory cells. Th2 cells become activated and produce a variety of cytokines and chemokines that, in turn, trigger recruitment of inflammatory cells, including eosinophils, and various other responses such as mucous production.

encounter, professional Ag presenting cells (APCs), of which dendritic cells (DCs) are the most powerful, engulf and process the allergen, traffic to draining lymph nodes and present allergen fragments (usually peptides) to circulating T and B cells (14). Recognition of allergen peptides by these cells, in conjunction with additional co-stimulatory signals from the APC, triggers T/B cell activation, differentiation and proliferation. For T cells, this typically involves acquisition of the aforementioned type-2 phenotype (12, 14). In turn, Th2 cells aid B cells to undergo Ig isotype switching by producing the cytokines IL-4 and IL-13, thereby facilitating B cell production of allergen-specific IgE and IgG₁ (15, 16). Circulating IgE quickly binds to receptors (FcεRI; the high affinity IgE receptor) on the surface of mast cells, a cell type located in the respiratory submucosa, and is ready to engage an immune response whenever that particular allergen is reencountered (16). Importantly, the initiation and successful execution of these events is largely thought to depend on the conditioning imposed by the lung microenvironment on the APC at the time of allergen encounter. The signals and mechanisms responsible for driving sensitization and the development of allergic immunity, as opposed to tolerance, remain the subject of intense investigation and will be discussed, in brief, in a following section. Of significance, sensitization, although required, does not necessarily imply the occurrence of disease/clinical symptoms; as is clear from the disparity in the proportion of the population that is sensitized versus that with disease.

Once sensitized, elicitation of the allergic airway response occurs upon subsequent allergen exposure. Allergen binding to, and crosslinking of, IgE on mast cells results in their activation and the release of various granule-associated and newly generated

mediators (16). Early mediators such as histamine, prostaglandins, and the cysteinyl leukotrienes stimulate smooth muscle contraction and increase local blood flow, vascular permeability, and the expression of adhesion molecules, which act in a cooperative manner to promote leukocyte recruitment (known as the ‘early’ allergic response; occurs within minutes), while cytokines and chemokines such as CC-chemokine ligand (CCL)-2, -5, -11, -17, -20, -22, and -24, direct macrophages, eosinophils, and T/B cells into the lung, concomitantly priming them for further mediator release (known as the ‘late’ response; occurs within hours-days) (12, 13). Recruitment of eosinophils is highly dependent on the production of IL-5, the major source of which is the Th2 cell (17). IL-5 induces eosinopoiesis and mobilization of eosinophils from the bone marrow and, in addition, aids in the activation and survival of eosinophils in the lung (17). Among the various mediators produced by the inflammatory cells are cytokines and chemokines, such as those already mentioned above; members of the growth factor family, namely, transforming growth factor (TGF)- β , platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF); as well as several toxic mediators, including proteolytic enzymes and reactive oxygen and nitrogen species (12, 13). Together, these factors not only augment leukocyte activation and recruitment, but, also cause damage and induce responses from numerous structural cells (e.g. smooth muscle contraction, mucous production by epithelial cells, synthesis of extracellular matrix components by fibroblasts) (12, 13). The combined effect of these processes culminates in the clinical symptoms that are characteristic of allergic asthma such as shortness of breath, chest tightness, coughing and wheezing. As many of the allergens that people are most commonly sensitized to are

ubiquitous in nature, it is intuitive that the natural evolution of asthma is to become a chronic process.

Central Aim

In the context of allergic asthma, ‘chronicity’ can be envisioned in different ways: as an ongoing airway inflammatory response elicited by continuous allergen exposure, exemplified by exposure to perennial CEAs such as house dust mite (HDM) or, alternatively, as recurring episodes of allergic airway inflammation induced by intermittent allergen exposure, perhaps best represented by exposure to seasonal CEAs such as ragweed. In either case, the chronic nature of this disease is likely to impart unique consequences that may have otherwise not existed in the context of an acute response. The central aim of this thesis is to investigate some of the potential consequences of chronic allergen exposure and persistent allergic airway inflammatory responses on disease pathogenesis. Of particular interest is the impact of chronic airway inflammation, elicited by continuous exposure to one aeroallergen, on the development of sensitization to other newly encountered allergens. This topic informs one of the bodies of work presented in this thesis (Chapter 2). The chronicity of the inflammatory response in asthma is also likely to impose unique stresses on the structure of the airways. Indeed, the consequence of this stress is thought to be the development of various structural alterations to the airway walls, collectively termed *airway remodeling*. In asthma, characteristic remodeling changes include thickening of the airway smooth-muscle layer, increased extracellular matrix (ECM) deposition beneath the epithelium and increased numbers of blood vessels and mucous-secreting goblet cells (18, 19). In comparison with our knowledge of the processes that

govern allergic inflammation much less is known about the mechanisms by which the inflammatory response may drive the development of airway remodeling. Thus, the contributions of the central growth factor TGF- β , and the hallmark allergic cell type, the eosinophil, in the generation of allergic airway remodeling are examined in the second and third bodies of work presented in this thesis (Chapters 3 & 4).

The remaining sections of this Introduction are structured to provide a thorough overview of the factors and mechanisms thought to influence the development of allergic airway sensitization and remodeling. The latter will be particularly focused on the current state of knowledge regarding the roles of TGF- β and eosinophils in remodeling. As animal models of allergic airway disease are a fundamental component of the work presented in the proceeding chapters, a brief outline of the models most relevant to this thesis is discussed immediately below.

Modeling Allergic Asthma

That humans are the subjects of ultimate interest, as it pertains to allergic asthma research, underscores the importance of studying *human* allergic asthma. However, practical and ethical considerations have largely limited asthma research in humans to *ex vivo/in vitro*- and observational-based investigations. Undoubtedly, these studies have furthered our knowledge of the action of various allergy-associated mediators, aided in the development of novel therapeutics, and helped to identify factors that influence the development of asthma. Nonetheless, the nature of these studies ultimately restricts in depth dissection of the molecular and cellular processes involved in the allergic response.

In this regard, animal models of allergic disease have enabled researchers to conduct detailed mechanistic experiments that are simply not feasible in humans, using systems that reliably reproduce features of the human condition. As a result, experimental models of allergic disease have contributed immensely to our understanding of disease pathogenesis. Below is a description of the two experimental systems most pertinent to the work presented and discussed in this thesis.

Conventional OVA-based Models of Allergic Airway Disease

Numerous studies have been conducted in animal, usually mouse, models of allergic airway disease. Early initiatives were largely focused on recapitulating some of the key features of allergic asthma: inflammation and AHR. The large majority of studies have utilized what is commonly referred to as ‘conventional’ models of allergic airway disease (refer to Figure 2A). Barring minor variations in timing and/or dose, these models involve intraperitoneal injection of chicken egg ovalbumin (OVA), a surrogate allergen and innocuous protein, in conjunction with a chemical adjuvant (usually aluminum-based) as a means of achieving allergic sensitization; such manipulations are necessary as airway exposure to OVA alone leads to tolerance not allergic sensitization (20, 21). This is followed by a short challenge phase where animals are exposed to OVA via the airways in order to elicit a consistent and vigorous allergic-type inflammatory response that is associated with the presence of Th2-associated cytokines, OVA-specific IgE, mucous production and AHR. Hence, these models have enabled researchers to extensively dissect and characterize the many immune-inflammatory components and their relation to airway

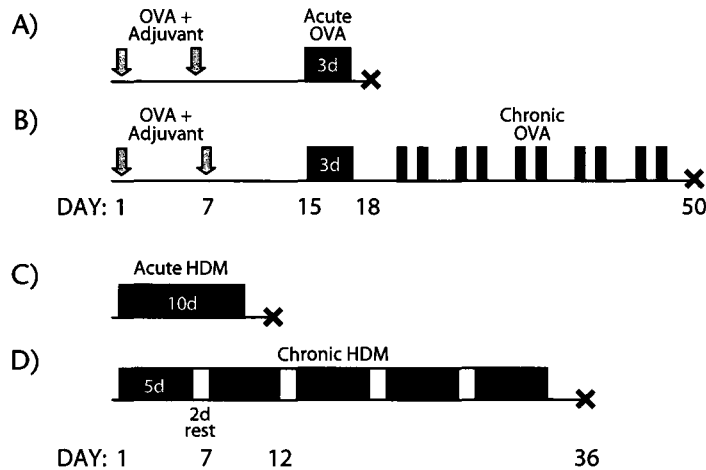


Figure 2. *Mouse models of allergic airway disease.* Representative protocols of A) acute and B) chronic conventional OVA-based models. Sensitization is achieved, in both acute and chronic protocols, by intraperitoneal injection of OVA adsorbed to a chemical adjuvant, usually aluminum-based. Allergic airway responses are subsequently elicited by subjecting sensitized mice to a short (e.g. 3 day) OVA airway challenge; acute protocol (A). Airway remodeling is induced by subjecting sensitized mice to an extended OVA exposure phase (e.g. 2 days/week for 5 weeks); chronic protocol (B). C) Acute HDM-based model; mice are subjected to 10 consecutive days of respiratory (intranasal) exposure to an HDM extract. Allergic airway responses are examined on day 12. D) Chronic HDM-based model; severe allergic airway responses including airway remodeling are elicited by subjecting mice to HDM exposure 5 days/week for 5 weeks.

dysfunction. Research related to aspects of the chronic asthmatic response including airway remodeling, which do not develop in these acute conventional protocols, require an extended exposure phase whereby the initial challenge is followed by several weeks of intermittent airway OVA exposure (two–three OVA challenges/week; refer to Figure 2B). These ‘chronic’ models of disease although successful in generating airway remodeling typically lead to the resolution of the immune-inflammatory response and the loss of type-2 responsiveness to OVA (22).

Notably, sensitization in humans does not involve the manipulations employed in conventional models, i.e. intraperitoneal injection of Ag along with a chemical adjuvant. Thus, the biggest limitation of these models is that they actually bypass many of the natural

processes and requirements for allergic sensitization, effectively prohibiting the study of this phenomenon. Moreover, the potential contributions of the respiratory mucosa and, CEAs are ignored.

HDM-based Models of Allergic Airway Disease

In 2000, our laboratory embarked on a process to develop mouse models of allergic airway disease that employed CEAs and established sensitization by engaging the respiratory mucosal microenvironment (refer to Figures 2C and D). In this regard, we demonstrated that ten consecutive days of respiratory exposure to an HDM extract, in the absence of exogenous adjuvants, was sufficient to elicit long-lived Th2-polarized sensitization that was also accompanied with systemic and local markers of type-2 immunity, eosinophilic airways inflammation, and AHR (23). We also developed a model of chronic HDM-induced allergic airway disease that involved continuous HDM exposure for several weeks, specifically five consecutive days of HDM exposure/week for five weeks (24). Under these conditions, we showed that in addition to the generation of robust Th2-polarized immunity continuous respiratory HDM exposure elicited maintained eosinophilic airway/lung inflammation and HDM-specific responsiveness, marked airway remodeling and profound AHR. This chronic HDM-based model has fostered novel explorations and was instrumental to the work presented in Chapters 2-4 of this thesis.

Factors Influencing the Development of Allergic Sensitization and Asthma

The current thinking is that asthma, and other allergic diseases, arise due to a complex interplay between many environmental and genetic factors (25). More specifically, that the set of environmental factors to which a person may encounter during early-life, interacts with particular genotypes thus influencing the development of the immune system in a manner that either protects against or predisposes towards the generation of allergic disease.

Genetic Factors

The influence of a genetic component on the development of allergic diseases is well-recognized (26, 27). Indeed, familial aggregation in asthma and the demonstration that monozygotic twins show higher concordance rates than dizygotic twins for markers of allergic sensitization (serum IgE) and self-reported asthma, strongly support this notion (28, 29). However, delineating the precise contribution of genetic elements to the development of asthma has proven difficult. A number of genome-wide searches have been conducted and various candidate genes have shown linkage and association of asthma-associated phenotypes to alleles of microsatellite markers and single nucleotide polymorphisms within certain cytokine and IgE regulating genes (25–27, 30–32). Yet, many of these associations remain controversial (e.g. (33) regarding CD14) and few, if any, have been shown to confer significant risk (26, 27, 33). Moreover, the relatively short period of time during which the prevalence of asthma rose makes it highly unlikely that this increase is attributable to population-wide genetic shift and, instead, underscores the

importance of environmental factors in the development of allergic sensitization and disease.

Impact of Inhaled Environmental Factors on Respiratory Mucosal Immune Responses

The respiratory mucosa is exposed to an overwhelming diversity of biological and non-biological agents. Of these, several have demonstrated the capacity to not only augment the asthmatic response in an already sensitized individual but, also, to facilitate *de novo* allergic sensitization and, subsequently, airway inflammation and lung dysfunction. As indicated earlier, respiratory exposure to an innocuous protein such as OVA results in the development of inhalation tolerance (20, 21). Immunologically, this state arises when DCs present Ag to lymphocytes in the absence of appropriate co-stimulatory signals (34, 35). Antigen recognition without such signals renders those Ag-specific lymphocytes anergic², or alternatively, results in their outright deletion from the lymphocyte pool (34, 35). In sharp contrast, inhalation of OVA in the context of an acute infection with respiratory syncytial virus (RSV), known to induce Th2-polarized immunity (36), subverts tolerance induction, and instead, facilitates the development of OVA-specific allergic airway disease (37, 38). Interestingly, this effect is not limited to Th2-inducing viruses as infection with Influenza A, a prototypical Th1-inducing virus, also prevents tolerance to OVA and drives OVA-specific Th2-polarized immunity (39-41). Notably, both RSV and influenza are common respiratory infections well known to significantly impact respiratory function in children during the first few years of life (42, 43). In addition, RSV has been strongly associated with the development of asthma in young children (6, 44). Furthermore, while

² A state of inactivation whereby a lymphocyte is nonresponsive to its cognate antigen.

‘high’ dose exposure to ubiquitously present bacterial-associated compounds, such as lipopolysaccharide (LPS) and peptidoglycan, have been shown to protect against the induction of allergic responses, experimental studies have demonstrated that, similar to some viral infections, ‘low’ dose exposure with OVA can, in fact, promote the development of robust OVA-specific allergic airway inflammatory responses (45-47).

The impact of various pollutants on lung function and the induction of allergic asthma have also been directly investigated (48, 49). Exposure to pollutants such as NO₂, O₃, and SO₂, either alone or in combination, has been consistently shown to trigger non-specific bronchial constriction and impairment of lung function in healthy subjects, with even greater effects being observed in asthmatics (48, 50, 51). Furthermore, in asthmatics, exposure to these pollutants significantly lowers the allergen provocation dose that induces AHR (52, 53). What is perhaps most alarming is the evidence attesting to the ability of such pollutants to facilitate *de novo* sensitization and subsequent allergic airway responses. Studies in animal models have reported that diesel exhaust particles (DEPs) can act as adjuvants to drive the development of allergic sensitization and airway immune-inflammatory responses to OVA upon coexposure (54, 55). Relatedly, human subjects nasally immunized with keyhole limpet hemocyanin (KLH) and concurrently exposed to DEPs developed *de novo* IL-4 and anti-KLH-specific IgE production, indicating that DEPs can facilitate allergic sensitization in humans as well (56). In addition to DEPs, ambient airborne particles, such as those collected from Ottawa dust, and both mainstream and secondhand tobacco smoke have all exhibited the capacity to prevent the induction of

tolerance to OVA and promote OVA-specific allergic mucosal sensitization, airway inflammation and, in some cases, AHR (57-59).

From the perspective of mechanism, the manner by which these biological and non-biological factors may exert these effects (sensitization, inflammation, etc.) is varied. Infectious agents and non-biologicals alike are able to attenuate ciliary function and increase epithelial permeability (60-66). As a result, they may foster the development of allergic responses by enhancing not only their own entry into the submucosal space but also the entry of any simultaneously inhaled antigen/allergen. Moreover, they can directly activate resident structural and immune cells by cleaving or binding to cell receptors. For instance, pattern-recognition receptors, such as toll-like receptors (TLRs), are specialized at recognizing pathogen-associated molecular patterns contained within microbial components (67, 68) and, are expressed by epithelial cells, macrophages and DCs. In a similar way, the aryl hydrocarbon receptor recognizes elements of environmental pollutants (69). Activation of such receptors triggers the production of numerous pro-inflammatory cytokines, notably granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-6, and IL-8, among others, and leads to DC activation and recruitment of innate immune cells such as neutrophils and monocytes/macrophages (67-69). While each distinct environmental agent may elicit a somewhat unique profile of cellular and molecular responses, the underlying mechanism that is thought to tie these agents together in their ability to promote allergic airway responses is their capacity to establish a lung microenvironment that is subversive with respect to tolerance and, instead, conducive to the induction of allergic sensitization. Indeed, it is becoming increasingly clear that the

immune status of the lung, at the time of allergen exposure, is a critical determinant of the ensuing immune response (tolerance vs. inflammation), and hence, the development of allergic airway disease.

Our view is that allergic airway disease develops when an allergen is encountered in the context of a lung microenvironment that has been conditioned to overcome the induction of tolerance, by any one or a combination of environmental exposures. Presumably, the 'threshold' of immune activation required to subvert tolerance is unique to each individual and influenced by various factors including genotype and prior immunological history. From this perspective, the increase in asthma prevalence could be attributable to increases in the amount of these environmental factors, i.e. greater exposure to pollutants, smoke, respiratory infection, etc., and/or due to decreased exposure to agents that may protect against allergic disease. This notion does not imply that aeroallergens are inherently unable to elicit allergic disease. On the contrary, the ability of at least some aeroallergens to directly engage the immune system and elicit immune responses such as proinflammatory cytokine production is well documented (70-72). In addition, respiratory exposure to some allergen extracts is, under certain conditions, sufficient to induce allergic airway immune-inflammatory responses in mice (23, 71, 73). While at least some component of these responses may be attributable to the non-protein elements of these extracts (74), certainly the direct involvement of allergens in this process has also been observed (72, 75-77).

That some aeroallergens can directly trigger immune responses and that the development of allergic disease is heavily influenced by the lung microenvironment are

concepts of fundamental importance to the work presented in Chapter 2. There we examined whether chronic allergic airway inflammation, that is, an established Th2-polarized environment, could drive sensitization and the development of allergic airway responses to a bystander antigen intrinsically unable to induce Th2 immunity on its own.

Factors Influencing the Development of Allergic Airway Remodeling

The development of an acute inflammatory response inevitably leads to some degree of tissue injury that is generally followed by repair and restoration to normal structure and function. Conversely, the chronic nature of the inflammatory response in allergic asthma forces reparative responses to occur under conditions of ongoing or repetitive injury. The end result of this is thought to be the generation of an altered or ‘*remodeled*’ airway structure. This notion is strongly supported by many studies in experimental animal models that have consistently documented the development of remodeling/fibrosis in response to chronic allergic inflammation (24, 78, 79). Further examination has demonstrated that airway remodeling develops specifically in response to chronic type-2-polarized, but not type-1, inflammation, illustrating that persistent inflammation, *per se*, is an insufficient requirement (80, 81). Notably, the observation that airway remodeling can occur in asthmatic children as young as 3 years of age has led some to question the role of inflammation in this process (82-84). However, the lack of detailed knowledge pertaining to the immunological history of these children, combined with the ‘snapshot’ nature of these studies weakens this doubt.

Characteristics and Clinical Impact of Airway Remodeling

The remodeled airway wall of asthmatic patients and mice subjected to chronic models of allergic airway disease is characterized by an overall increase in thickness. This thickening comes at the expense of increases in essentially all major structural components: muscle, matrix, epithelium/mucous glands, and vasculature. Increases in smooth muscle mass, on account of smooth muscle cell (SMC) hypertrophy and hyperplasia, are regularly observed in patients with asthma (85-88) and although studies have demonstrated some heterogeneity among asthmatics this increase has been observed in both the large and small airways (85, 86, 88). The increase in smooth muscle may be due to the action of several processes including growth and proliferation of SMCs induced by cytokines, growth factors, and other inflammatory mediators (89-91); work related increases in smooth muscle size stemming from repeated bronchospasm; or from a reduction or loss of smooth muscle inhibitory mechanisms (18). Similarly, increases in both the size and number of mucous glands are also commonly observed in asthmatics (85, 92). These glands are normally limited to cartilage-associated airways although in patients with asthma they have also been observed in peripheral bronchioles. Overall, mucous glands constitute a higher proportion of the submucosal space in asthmatics compared with non-asthmatics, at least in cases of fatal asthma (85, 92, 93). Greater numbers of mucus producing epithelial goblet cells are also present (94, 95). The characteristic thickening of the basement membrane ECM layer in the asthmatic bronchi, often referred to as 'fibrosis' of the airways, results from increased deposition of collagen, mainly types I, III and IV, and the glycoproteins tenascin and fibronectin (96-99). All resident lung structural cells contribute to the thickened ECM in the remodeled airway by secreting these various ECM components.

Greater immunoreactivity of other ECM-associated components such as laminin, decorin and versican has also been described in the airway subepithelial matrix of asthmatics relative to control subjects, albeit somewhat inconsistently (19). Increased blood vessel area is also a common feature of the thickened airway wall in asthma. Generally, studies have noted that vessels are larger and greater in number in asthmatics compared to normal non-asthmatic individuals (88, 100).

Presumably, the thickened airway wall along with increased mucus production encroach on the airway lumen, thus reducing airway caliber, increasing airway resistance and, hence, triggering clinical symptoms. Certainly, numerous, but not all (19, 101), CT- and morphometric-based human studies have reported that increased airway thickening is associated with various clinical features of asthma, including the expression of symptoms, impaired lung function, and increases in AHR and disease severity overall (19, 102-105). Importantly, mathematical modeling-based studies have confirmed that the remodeled airway does have a functional impact (106, 107). In addition, experimental studies have shown that mice subjected to chronic allergen challenge models display persistent AHR even many weeks after allergen exposure has ceased and at a time when airway inflammation, but not remodeling, has resolved (24, 108). Nevertheless, the issue is complex; for example, some reports have shown that the thickened airway is not commonly associated with narrowing of the airway lumen (105, 109). Calculated data suggest that the thickened airway wall may increase airway resistance only marginally (~10%) under resting conditions (110, 111). However, additional studies indicate that shortening of the smooth muscle, such as during times of mild-severe symptoms, greatly

amplifies the impact of airway remodeling on lumen size and, likely, airway function (106, 107, 110, 111). Moreover, the precise functional consequences of individual remodeling-associated alterations remain largely unclear (19). There is also considerable debate about the possibility that certain remodeling-associated changes exert beneficial effects (19, 112). For instance, an inverse relationship was observed between basement membrane thickness and airway responsiveness (113), and increases in ECM around smooth muscle bundles may prevent or limit smooth muscle thickening during contraction (114). Ultimately, although a number of issues remain unresolved, it seems evident that airway remodeling, or at least certain elements of it, imposes a significant deleterious clinical and functional impact.

The TGF- β –Eosinophil Paradigm in Allergic Airway Remodeling

Our knowledge of the mechanisms that govern the pathogenesis of airway remodeling remains limited at this time, but is growing rapidly. Many mediators have been implicated in the development of airway remodeling on account of their ability to influence reparative/fibrotic responses. These include, among others, enzymes such as matrix metalloproteinases (MMPs), cytokines including IL-11, oncostatin M, and the Th2-affiliated cytokines IL-5 and IL-13, and members of the growth factor family, notably, TGF- β , PDGF, and VEGF (115-122). Among these, TGF- β is considered to be the most influential as it is a potent regulator of the growth and function of most structural cells and is thought to play a central role in the regulation of the ECM in the lung and elsewhere.

Many, if not all, cells in the lung can produce and respond to TGF- β . However, in the context of allergic asthma epithelial cells, fibroblasts, macrophages and eosinophils are likely to be of greatest relevance and, of these, eosinophils are primarily considered to be the major cellular source of TGF- β and, hence, a central mediator of airway remodeling. In general, the findings in support of this are that i) eosinophils abundantly express TGF- β *in vitro* upon activation (123, 124), ii) TGF- β expressing eosinophils are often observed in the lungs of mice subjected to models of allergic airway disease (125-127) and, in human asthmatic lungs (128-131), with some studies reporting that >90% of the TGF- β expressing cells in bronchial biopsies from asthmatic subjects are eosinophils (132) and iii) eosinophils have been directly shown to mediate airway remodeling in some OVA-based experimental investigations (115, 133). This seemingly compelling evidence has led to the emergence of the TGF- β – Eosinophil paradigm which, in essence, proposes that eosinophil-derived TGF- β production (and/or activation) is a major mechanism that drives the development of airway remodeling in allergic asthma. Given the implications of this hypothesis to our understanding of how remodeling develops and, consequently, how it may be treated, a detailed examination of the evidence pertaining to the role of TGF- β and eosinophils in allergic remodeling is warranted and, thus, provided below.

TGF- β in Tissue Repair and Allergic Airway Remodeling

Since its discovery over twenty-five years ago (134, 135), it has become apparent that TGF- β is one of the most pleiotropic cytokines in mammals. Indeed, many

investigations have revealed that TGF- β plays integral roles in complex processes including embryonic and post-natal development, tissue repair, and immunity and critically influences basic cellular activities such as migration, proliferation, and apoptosis. While TGF- β is not the only mediator involved in tissue repair, it is widely considered to be the most powerful regulator of reparative responses (136, 137). In the lung, studies suggest that TGF- β orchestrates the ECM by influencing at least three different processes: i) the synthesis of various ECM components such as collagen and fibronectin by fibroblasts, airway smooth muscle cells and epithelial cells, ii) the expression of matrix-degrading enzymes such as MMPs, and iii) the levels of anti-proteases such as tissue inhibitor of metalloproteinases (TIMPs) (136, 137). *In vitro*, studies have demonstrated that TGF- β can induce smooth muscle growth (138), stimulate mesenchymal cells, including lung fibroblasts and airway SMCs, to divide (139-141) and trigger fibroblasts to undergo differentiation into myofibroblasts (142, 143), a cell type thought to play a critical role in wound repair and airway remodeling (144). Moreover, TGF- β may further contribute to reparative/fibrotic responses by stimulating various cells to produce additional pro-reparative molecules such as PDGF, VEGF and CTGF (connective tissue growth factor), among others (139, 145, 146). Importantly, delivery of an adenoviral vector engineered to overexpress TGF- β in the lungs of rats was sufficient to induce the development of robust pulmonary fibrosis (147). Therefore, on the basis of its functional capacities alone, TGF- β seems capable of driving many of the structural alterations that are typically observed in the remodeled airway wall of asthmatics.

The signaling pathways mediating the effects of TGF- β are complex and involve a number of molecules (136). A pathway of increasingly recognized importance includes a family of signal transduction proteins referred to as ‘Smad’ (refer to Figure 3). Bioactive TGF- β signals through transmembrane serine/threonine kinase receptors designated TGF-

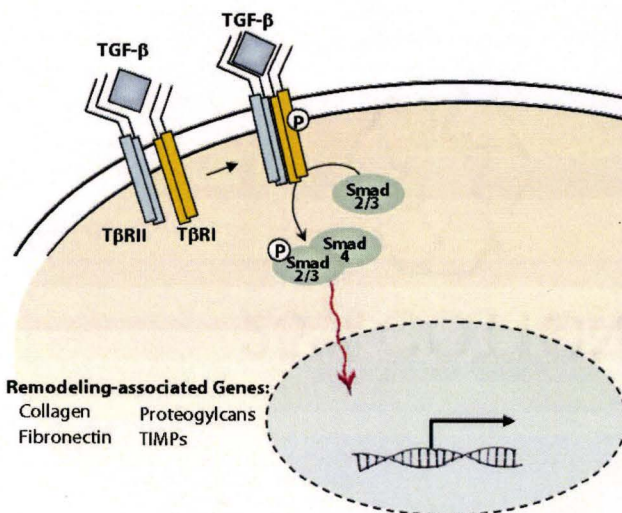


Figure 3. Schematic of the TGF- β -induced Smad signaling pathway. Refer to text for description of the figure.

β type I (T β R1) and type II (T β R2) receptors. TGF- β binding to T β R2 is, then, followed by the recruitment and activation (i.e. phosphorylation) of T β R1. Activation of T β R1 leads to phosphorylation of Smad2 and Smad3 which, then, bind to Smad4; this complex translocates to the nucleus to activate an array of TGF-

β -responsive promoters (148). Of significance, this pathway appears to play a fundamental role in the signal transduction pathways associated with wound healing and fibrosis (149, 150). Indeed, T β R1 inhibition has been shown to prevent the induction and progression of TGF- β -induced pulmonary fibrosis (151) and, moreover, Smad3-deficient mice are less able to develop TGF- β -induced lung and cutaneous radiation-induced fibrosis (152, 153).

A number of studies have investigated the expression of TGF- β and its relationship with airway remodeling in human asthmatics. In this regard, TGF- β levels were found to be elevated in the bronchoalveolar lavage (BAL) of stable atopic asthmatics compared with

control subjects (130). Similarly, increased amounts of TGF- β on both the level of mRNA and protein have been observed in several bronchial biopsy studies of moderate-severe asthmatic patients (128, 129, 131, 132, 154, 155) although, notably, not all such studies have documented differences in TGF- β expression among asthmatics and controls (156). Significant correlations have been noted between basement membrane thickness and the number of TGF- β expressing epithelial or submucosal cells in asthmatic patients versus normal subjects (131) and, in agreement with this, a separate study reported that basement membrane thickness was also significantly associated with the presence of activated Smad2 (157), a marker of TGF- β activity. Relatedly, TGF- β expression has also been shown to correlate with the number of fibroblasts in the remodeled airway wall of asthmatics but not in control subjects (158). Together, these findings have been used to construct the notion that TGF- β is involved in the development of airway remodeling although, admittedly, the correlative nature of the above studies considerably limits their significance.

The role of TGF- β in remodeling has been directly investigated in at least four animal studies employing OVA-based experimental models of allergic airway disease. In general, elevated TGF- β expression is a common feature of allergic airway responses in animals subjected to various models of allergic airway disease (125-127, 159). Using an anti-TGF- β antibody (Ab) that interferes with the function of all three mammalian TGF- β isoforms, McMillan *et al.* neutralized TGF- β activity throughout the chronic exposure phase of their OVA-based model and observed considerable decreases in the number of

proliferating airway SMCs and significant, but partial, reductions in airway mucus production and peribronchial ECM deposition relative to mice treated with a control Ab (118). Likewise, treatment with a TGF- β 1-specific blocking Ab was also shown to substantially reduce the increases in peribronchial collagen observed in mice subjected to a somewhat shorter OVA-exposure protocol (160). In addition, Smad3-deficient mice conventionally sensitized and repeatedly challenged with OVA demonstrated a complete reduction in the number of bronchial-associated myofibroblasts and dramatic decreases in total lung collagen relative to wild-type (WT) OVA-exposed mice (161). This was further accompanied by partial reductions in the thickness of the peribronchial smooth muscle layer and in mucus production. Finally, sensitized rats treated during OVA exposure with a T β R-I kinase inhibitor that interferes with the activation of the Smad-signaling pathway displayed markedly lower epithelial and smooth muscle cell proliferation and goblet cell hyperplasia compared to positive controls (162). The impact of this treatment on more robust markers of airway remodeling, including ECM deposition and smooth muscle thickness were not examined. Collectively, these experimental studies clearly document a central role for TGF- β in the development of OVA-based allergic airway remodeling and, therefore, support the notion that TGF- β may be an important mediator of remodeling in humans.

Eosinophils in Allergic Airway Remodeling

The presence of eosinophils in the lungs of allergic asthmatics has been described for over a century. Eosinophils are granulocytic cells produced in the bone marrow from pluripotent stem cells under the influence of several transcription factors and cytokines,

notably, GATA-1 and IL-5, respectively (17). Normally, eosinophils predominately reside in the gastrointestinal tract and typically comprise less than 3% of blood leukocytes. While these cells were believed to function mainly in host defense against parasites it is now recognized that eosinophils are multifunctional leukocytes that can participate in processes as diverse as mammary gland development, estrus cycling, immunity to viral infections and allergic inflammation (17, 163). The spectrum of receptors expressed on the surface of eosinophils along with the diversity of mediators they produce (164) reflect their varied capabilities and illustrate their potential to exert many effects.

Traditionally, eosinophils have been viewed as destructive effectors, largely on account of their demonstrated ability to produce toxic granule proteins (165-168). However, more recently, it has been noted that eosinophils may also contribute to tissue repair responses and, hence, in the context of allergic asthma, airway remodeling. *In vitro* experiments have demonstrated that human bronchial epithelial cells co-cultured with subcytotoxic amounts of eosinophil peroxidase (EPO) or major basic protein (MBP) responded by upregulating the expression of PDGF, endothelin-1 and MMP-9 (169), all of which have been implicated in the repair/remodeling response. Furthermore, eosinophils can produce a variety of other pro-fibrotic factors, notably, EGF (epidermal growth factor), FGF (fibroblast growth factor), and TGF- β (164) and, in this way, may further drive repair/remodeling by influencing the function, differentiation and proliferation of lung structural cells.

Initial investigations into the involvement of eosinophils in the generation of allergic airway remodeling abolished pulmonary eosinophilia, indirectly, by impairing IL-5 function. Reports by Blyth *et al.* and Tanaka *et al.* both noted the complete and selective ablation of airway eosinophilia following treatment with anti-IL-5 in mice subjected to short-term and chronic OVA-based exposure models, respectively (120, 170). Interestingly, this was associated with a complete reduction in subepithelial matrix deposition. Moreover, the Tanaka study confirmed these findings using IL-5-receptor- α -deficient mice (120). Similarly, treatment with anti-IL-5 during the final two weeks of a six week OVA-based exposure protocol markedly inhibited the eosinophilic infiltrate and this effect was associated with a significant, but partial, decrease in subepithelial fibrosis (171). In yet a separate study, IL-5-deficient mice that were conventionally sensitized to OVA and subjected to a chronic exposure protocol displayed considerable decreases in the thickness of the smooth muscle layer and the accumulation of peribronchial collagen, types III and V, and moderate reductions in total lung collagen levels and mucous production relative to WT OVA exposed control mice (115). Importantly, eosinophils were virtually absent from the BAL of OVA-exposed IL-5-deficient, but not WT, mice whereas the numbers of peribronchial macrophages and helper (CD4⁺) T cells and the levels of BAL IL-13 were comparable between these two groups. Two additional studies conducted in IL-5-deficient mice that also employed conventional chronic OVA-based models of allergic airway disease disagree. Indeed, although Leigh *et al.* documented decreased α -smooth muscle actin in OVA-exposed IL-5-deficient mice compared to WT controls, no reduction in subepithelial collagen deposition was noted (80). Similarly,

despite the absence of eosinophils, and less overall inflammation in the airway wall, Foster *et al.* observed increases in peribronchial collagen deposition and in the thickness of the airway epithelium in OVA-exposed IL-5-deficient mice that were equivalent to that measured in WT sensitized and challenged controls (172). Several explanations have been proposed in an attempt to resolve these discrepancies although, ultimately, none have proven adequate (discussed in Chapter 4). Noteworthy, the use of IL-5-based interference strategies, as a means of abrogating eosinophils, may further complicate interpretations of the above findings as IL-5 is known to be involved in other processes that may impact airway remodeling in an eosinophil-independent manner (173, 174).

In order to directly ascertain whether eosinophils are involved in the development of airway remodeling, and to avoid any potential ambiguities associated with IL-5-based approaches, Humbles *et al.* utilized a strain of mice, Δ dblGATA, that were engineered to be selectively deficient in eosinophils. Interestingly, relative to WT mice, GATA eosinophil-deficient mice subjected to a chronic OVA-based exposure model were largely protected from remodeling (133). More specifically, OVA-exposed GATA mice displayed considerable decreases in the number of total and proliferating airway smooth muscle cells and substantially less total lung collagen than OVA-exposed WT controls. Thus, although not without controversy, the bulk of the evidence from OVA-based studies suggests that eosinophils do participate in the development of the remodeled airway.

Role of TGF- β and Eosinophils in HDM-induced Allergic Airway Remodeling

We have been interested in investigating the mechanisms by which chronic allergic airway inflammation may influence the generation of remodeling and, specifically, the role of TGF- β and eosinophils in this process. However, in contrast to the studies that have addressed this issue (discussed above), in Chapters 3 and 4 we examine these matters in a model of chronic allergic airway inflammation induced by respiratory exposure to house dust mite allergens.

Objectives

The research presented in Chapters 2, 3, and 4 advance our understanding of the consequences of chronic allergic inflammation, a cardinal feature of allergic asthma, on sensitization and remodeling and explore the mechanisms by which these effects may be mediated.

The first manuscript in this thesis (Chapter 2) sought to investigate the impact that a lung microenvironment conditioned by chronic Th2-polarized inflammation would have on the immune response to a novel inhaled innocuous antigen. To this end, we subjected mice to the chronic HDM-exposure model and concurrently administered OVA, an Ag intrinsically unable to induce allergic responses on its own. We first determined whether co-exposure was sufficient to drive OVA-specific sensitization by examining systemic markers of OVA-specific adaptive immunity, including Th2-associated cytokine and immunoglobulin production. We subsequently explored whether HDM/OVA co-exposure was sufficient to induce bona fide allergic responses to OVA

upon reencounter. To assess this, we allowed mice that were concomitantly exposed to HDM and OVA to rest, in order for the initial inflammatory response to subside, and then examined the inflammatory and functional responses elicited in these mice following *in vivo* rechallenge with OVA alone.

The second and third manuscripts presented in this thesis (Chapters 3 and 4) investigate a potential mechanism by which chronic allergic inflammation may promote the development of airway remodeling. They investigate the hypothesis that persistent airway eosinophilia drives remodeling via the actions of TGF- β .

In the second manuscript (Chapter 3), we examine the requirement for TGF- β in the development of HDM-induced allergic airway remodeling. This project was grounded in the large body of literature attesting to TGF- β 's involvement in tissue repair responses and on an initial observation that the kinetics of HDM-induced TGF- β induction preceded and paralleled the development of the remodeled airway. The investigation proceeded along two lines. The first, involved the use of a pan-neutralizing anti-TGF- β Ab to block TGF- β function in mice, not only in the context of continuous HDM-exposure, but also, in the context of a novel intermittent HDM model that involved multiple, short, HDM rechallenges that were separated by rest periods. The second involved subjecting Smad3-deficient mice to the chronic HDM-exposure model. In both cases, the impact of interfering with TGF- β function on airway remodeling was evaluated by morphometric assessment of 3 relevant markers: goblet cell hyperplasia/mucus production, smooth muscle thickening, and subepithelial collagen deposition. Given that

TGF- β is also a powerful negative regulator of inflammation the immunological and functional consequences of blocking TGF- β were also investigated.

The third manuscript builds on the work of the previous chapter. Here, the role of eosinophils in the generation of HDM-induced remodeling is comprehensively examined. To this end, three separate methods, comprising two distinct strategies, were employed: an anti-CCR3 monoclonal Ab that specifically depletes eosinophils and both strains of eosinophil-deficient mice, Δ dblGATA and *PHIL*. Preliminary experiments were conducted in order to establish an optimal anti-CCR3 dosing regimen, which was then used to deplete eosinophils in mice subjected to the chronic HDM exposure model. The GATA and *PHIL* lines of eosinophil-deficient mice were similarly exposed to HDM. The impact of eosinophil ablation on remodeling was again assessed by morphometric analysis of peribronchial collagen deposition, smooth muscle thickness and goblet cell hyperplasia. We further evaluated splenocyte Th2-cytokine production, serum levels of HDM-specific IgE, inflammation in the BAL, activation state and numbers of lung Th2 cells, and AHR in order to determine the contribution of eosinophils to other components of the allergic response including adaptive immunity, inflammation and airway function.

— Chapter 2 —

House Dust Mite Facilitates Ovalbumin-specific Allergic Sensitization and Airway Inflammation

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See *Preface* for details regarding authorship.

Summary and Central Message: This article examines how chronic allergen exposure and persistent airway inflammation may impact the development of allergic sensitization to newly encountered antigens. We asked whether HDM, a bone fide allergen able to elicit allergic immune responses in mice on its own, could establish a lung microenvironment that would promote the development of allergic responses to other antigens, intrinsically unable to do so. To test this we exposed mice to an HDM extract concurrently with OVA, an innocuous antigen, for five weeks and then allowed mice to rest. We found that mice rechallenged to OVA alone, many weeks later, mounted a robust Th2-polarized immune inflammatory response in the lung that was associated with marked increases in AHR. Thus, these data demonstrate that chronic HDM exposure can subvert inhalation tolerance and facilitate the development of allergic sensitization and the full ‘asthmatic’ phenotype towards an innocuous antigen. Moreover, these findings highlight the importance of the lung immunological milieu in determining the type of immune response that develops following encounter with a foreign antigen.

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Rationale: Mouse models of allergic airway disease have greatly contributed to our understanding of disease induction and pathogenesis. Although these models typically investigate responses to a single antigen or allergen, humans are frequently exposed to a myriad of allergens, each with distinct antigenic potential. **Objectives:** Given that airway exposure to ovalbumin (OVA), a prototypic innocuous antigen, induces inhalation tolerance, we wished to investigate how this response would be altered if OVA were encountered concurrently with a house dust mite extract (HDM), which we have recently shown is capable of eliciting a robust allergic airway inflammatory response that is mediated, at least in part, by granulocyte-macrophage colony-stimulating factor. **Methods:** Balb/c mice were exposed daily to HDM (intranasally) followed immediately by exposure to aerosolized OVA for 5 weeks. To allow the inflammatory response elicited by HDM to subside fully, mice were then allowed to rest, unexposed, for 8 weeks, at which time they were rechallenged with aerosolized OVA for 3 consecutive days. **Measurements and Main Results:** At this time, we observed a robust eosinophilic inflammatory response in the lung that was associated with an increase in bronchial hyperreactivity. Moreover, we documented significantly elevated serum levels of OVA-specific IgE and IgG, and increased production of the Th2 cytokines interleukin 4 (IL-4), IL-5, and IL-13 by splenocytes stimulated *in vitro* with OVA. **Conclusion:** Our data demonstrate the potential of a potent allergen such as HDM to establish a lung microenvironment that fosters the development of allergic sensitization to otherwise weak or innocuous antigens, such as OVA.

Keywords: allergic sensitization; allergy inflammation; lung; mouse

Mucosal surfaces are under constant exposure to a diverse array of biological and nonbiological entities, each with a distinct pathogenic potential. To survive, the immune system has evolved, over millions of years, sophisticated mechanisms to determine the type of immunologic response that each warrants. However, because humans are exposed simultaneously to more than one agent, it is likely that concurrent exposure has an impact on the response the immune system evolved for each single entity. We

wished to explore this concept in the context of aeroallergen exposure.

Aeroallergens comprise a vast collection of nonreplicating entities, with diverse immunogenic potential capable of inducing specific immune-inflammatory responses (1, 2). Whether, and how, they facilitate allergic sensitization and airway inflammation remains a subject of intense research. A great deal has been learned through experimental modeling of aeroallergen exposure, much of it using ovalbumin (OVA) as a surrogate allergen. Because OVA is an archetypic innocuous antigen, and mucosal exposure to aerosolized OVA alone results in inhalation tolerance (3–5), conventional modeling has relied on the introduction of OVA into the peritoneum in conjunction with an adjuvant, generally aluminum hydroxide, to elicit allergic sensitization. In contrast, we have recently shown that intranasal exposure to a house dust mite (HDM) extract generated acute (6) as well as chronic (7) airway inflammation with the characteristic hallmarks of a Th2-type immune-inflammatory response. Because it is evident that humans are concurrently exposed to various aeroallergens, we were interested in investigating whether HDM could subvert the expected immunologic response to OVA. The issue is of relevance because there is a definite increase in the prevalence of allergic disease, including asthma (reviewed in Reference 8), and most of the increase has been observed with indoor allergens, notably HDM (8).

To our knowledge, only three studies have previously investigated the impact of concurrent allergen exposure on the development of allergic responses (9–11). The extent of the analysis in these three studies was limited to the assessment of serum levels of OVA-specific Th2-affiliated immunoglobulins after concurrent administration of a bona fide allergen with OVA. Thus, whether exposure to one allergen can facilitate the full development of the “asthmatic phenotype” in response to another remains to be determined.

To address this issue experimentally, we exposed mice to HDM concurrently with OVA for 5 weeks. Subsequently, mice were allowed to rest for 8 weeks, at which time they were then reexposed to aerosolized OVA alone. This *in vivo* OVA recall elicited a robust airway inflammatory response characterized by eosinophils and Th2 effector cells and was associated with bronchial hyperreactivity. Systemically, we documented increased serum levels of Th2-affiliated OVA-specific immunoglobulins and production of OVA-specific Th2-associated cytokines by splenocytes on *in vitro* recall. Collectively, these data demonstrate that HDM is able to subvert OVA’s intrinsic innocuous nature and to privilege a Th2 inflammatory response over the default tolerogenic bias (12).

METHODS

Animals

Female Balb/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Ottawa, ON, Canada). The mice were housed under specific pathogen-free conditions and maintained on a 12-hour light–dark cycle, with food and water *ad libitum*. All experiments

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described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada).

Sensitization Protocols

Allergen administration. HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in sterile phosphate-buffered saline at a concentration of 5.0 mg (protein)/ml, and 10 μ l was administered to isoflurane-anesthetized mice intranasally. After reconstitution, we evaluated the proteolytic activity of the HDM extract by zymography (as previously described in Reference 13), and observed that a single dose of HDM was indeed proteolytically active (data not shown). Mice that received OVA (Grade V; Sigma-Aldrich, Oakville, ON, Canada) were placed in a Plexiglas chamber ($\sim 10 \times 15 \times 25$ cm) and were exposed to aerosolized OVA (1% wt/vol in 0.9% saline) for 20 minutes. Aerosolized OVA was produced using a Bennet nebulizer with an airflow rate of 10 L/minute.

Short-term exposure. Mice were exposed daily to HDM, OVA, or HDM followed 1 to 4 hours later by OVA for 10 consecutive days.

Extended exposure. Mice were exposed daily to HDM, OVA, or HDM followed 1 to 4 hours later by OVA, for 5 consecutive days a week followed by 2 days of rest for a total of 5 weeks.

Rechallenge. After extended allergen exposure, mice were rested for a period of approximately 8 weeks at which time they were then rechallenged with OVA (as above) daily for 3 consecutive days.

Collection and Measurement of Specimens

Seventy-two hours after the last challenge, mice were killed and bronchoalveolar lavage (BAL) fluid, lungs, blood, and spleen were collected. BAL was performed as previously described (14). Total and differential cell counts were determined as previously described (14). Where applicable, after BAL, lungs were inflated with 10% formalin. Tissues were then embedded in paraffin, and 3- μ m-thick sections were cut and stained with hematoxylin and eosin. Peripheral blood was collected by retroorbital bleeding, and serum was obtained and stored at -20°C . See the online supplement for additional details on the methods used to make these measurements.

Splenocyte Culture

Spleens were harvested, and splenocytes were isolated and resuspended in complete RPMI at a concentration of 8×10^6 cells/ml and cultured in medium alone, or with medium supplemented with OVA (40 $\mu\text{g}/\text{ml}$). After 5 days of culture, supernatants were collected for cytokine measurement. See the online supplement for additional details on the methods used to make these measurements.

Cytokine and Immunoglobulin Measurements

Levels of interleukin 4 (IL-4), IL-5, IL-13, and IFN- γ were measured by ELISA using kits purchased from R&D Systems (Minneapolis, MN). Levels of OVA-specific IgE in the serum were measured using a previously described antigen-capture ELISA method (14), whereas OVA-specific serum IgG₁ and IgG_{2a} were measured by sandwich ELISA with OVA in the solid phase, as previously described in detail (15). See the online supplement for additional details on the methods used to make these measurements.

Lung Cell Isolation and Flow Cytometric Analysis of Lung Mononuclear Cells

Lung mononuclear cells were isolated as previously described (16) and subsequently stained with a panel of antibodies. Data were collected using a FACScan (Becton Dickinson, Franklin Lakes, NJ) four-color flow cytometer and analyzed using WinMDI software (Scripps Research Institute, La Jolla, CA). See the online supplement for additional details on the methods used to make these measurements.

Airway Hyperreactivity Measurements

Airway responsiveness was measured on the basis of the response of total respiratory system resistance to increasing intravenous (internal jugular vein) doses of methacholine as previously described (17). See the online supplement for additional details on the methods used to make these measurements.

Data Analysis

Data are expressed as mean \pm SEM. Results were interpreted using analysis of variance with a Tukey *post hoc* test, unless otherwise indicated. Differences were considered statistically significant when *p* values were less than 0.05.

RESULTS

Impact of HDM on the Induction of OVA-specific Immune Responses

Balb/c mice received daily administrations of HDM, OVA, or both HDM and OVA (HDM/OVA) for 10 consecutive days (Days 0–9) and were then killed approximately 72 hours (Day 12) after the last challenge (ALC; Figure 1A). Analysis of the BAL revealed no significant differences in the cellular profile of mice exposed to OVA alone compared with untreated (naive) mice (Figure 1B). In direct contrast, and as we have previously demonstrated (6), we observed significant increases in the number of total cells and eosinophils in the BAL of mice repeatedly exposed to HDM when compared with naive and OVA mice (Figure 1B). Furthermore, we noted significant increases in the BAL of HDM/OVA mice in terms of total cells and eosinophils versus naive and OVA mice with no differences between HDM/OVA and HDM mice (Figure 1B).

Because we were unable to differentiate between the OVA-elicited component of the inflammatory response from that

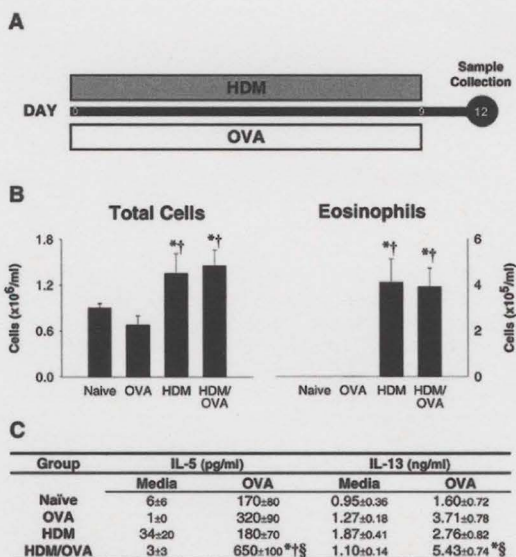


Figure 1. Effect of house dust mite (HDM) coexposure with ovalbumin (OVA) on the development of an OVA-specific immune inflammatory response. (A) Mice were exposed daily to OVA, HDM, or HDM and OVA (HDM/OVA) for a period of 10 consecutive days (Days 0–9) and then killed approximately 72 hours after the last challenge (ALC) (Day 12) at which time samples were collected for analysis. (B) Cellular profile in the bronchoalveolar lavage (BAL) fluid showing the number of total cells and eosinophils. (C) Production of interleukin 5 (IL-5) and IL-13 by splenocytes after *in vitro* stimulation with OVA or medium alone. $n = 5$ –9/group; $p < 0.05$ compared with *naive, † OVA, and ‡ HDM mice, respectively. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) *post hoc* test for C.

of HDM in the BAL of HDM/OVA mice, we examined the *in vitro* cytokine production by OVA-stimulated splenocytes as a means of assessing OVA-specific sensitization. We measured higher levels of the Th2-associated cytokines IL-5 and IL-13 (Figure 1C) produced by splenocytes isolated from HDM/OVA mice compared with naive and HDM groups after *in vitro* stimulation with OVA, although when compared with OVA mice, significance was only reached in the case of IL-5.

Impact of Extended Concurrent Allergen Exposure

OVA-specific markers of systemic sensitization immediately after extended concurrent exposure to HDM and OVA. Because 10-day exposure to HDM concurrently with OVA suggested the incipient generation of OVA-specific sensitization, we proceeded to examine the influence of HDM on the development of OVA-specific immunity in a protocol that involved prolonged exposure to both HDM and OVA. Thus, we exposed mice to daily administrations of HDM, OVA, or HDM/OVA on 5 consecutive days/week for a total of 5 weeks and then killed them shortly afterward (~ 72 hours ALC; Figure 2A). We observed significantly increased *in vitro* OVA-specific production of the Th2-associated cytokines IL-4 and IL-13 (Figure 2B), by splenocytes that were isolated from HDM/OVA mice compared with

naive or control mice. In addition, we did not observe any differences in OVA-specific production of the Th1-associated cytokine IFN- γ between any of the experimental groups (data not shown). Correspondingly, significant increases in the serum levels of OVA-specific IgG₁ were also observed in HDM/OVA mice compared with naive and control groups (Figure 2C). Analysis of the BAL revealed no significant differences in the differential cell profile of mice exposed to OVA alone versus naive (data not shown). In contrast, we observed significant increases in the total cell number of HDM/OVA mice compared with naive and OVA mice, which was characterized by increases in both mononuclear cells and eosinophils. No differences between HDM/OVA and HDM-alone mice were noted (data not shown).

OVA-specific recall responses after extended concurrent exposure: cellular profile in the BAL. To assess conclusively whether an OVA-specific immune response in the lung had developed on extended concurrent exposure to HDM and OVA, a group of mice were allowed to rest after 5 weeks of exposure for a period of approximately 8 weeks (Figure 3A), to allow the lung

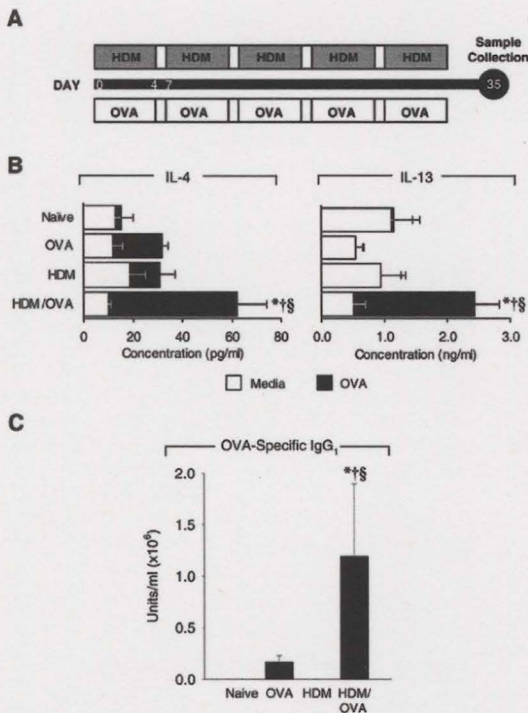


Figure 2. Th2-affiliated, OVA-specific splenocyte cytokine production and serum immunoglobulin levels immediately after extended allergen exposure. (A) Mice were exposed to OVA, HDM, or HDM and OVA (HDM/OVA) for 5 weeks as described in METHODS and killed approximately 72 hours ALC (Day 35) at which time samples were collected for analysis. (B) IL-4 and IL-13 production by splenocytes after *in vitro* stimulation with OVA (black bars) or medium alone (white bars). (C) Serum levels of OVA-specific IgG₁. n = 4–5/group; p < 0.05 compared with *naive, [†]OVA, and [‡]HDM mice, respectively. Statistical analysis was performed using one-way ANOVA with Fisher's LSD *post hoc* test for C.

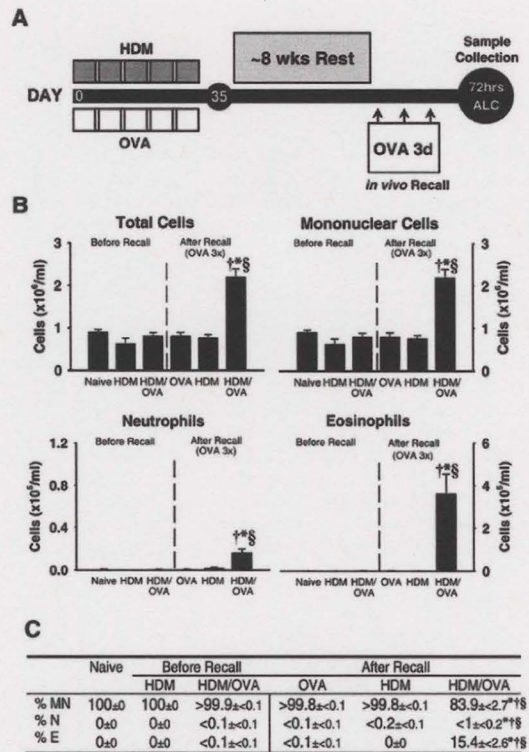


Figure 3. Eosinophilic airway inflammation after *in vivo* recall with OVA in the BAL of HDM/OVA mice only. (A) After extended allergen exposure, mice were allowed to rest for approximately 8 weeks and were then rechallenge with OVA alone, daily, for 3 consecutive days and then killed 72 hours later. (B) Differential cell analysis showing the number of total cells, mononuclear cells, neutrophils, and eosinophils in the BAL of mice killed: before *in vivo* OVA recall but after the rest period (Before Recall; left side of dashed line) or 72 hours after OVA recall (After Recall; right side of dashed line). (C) Percentage of mononuclear cells, neutrophils, and eosinophils in the BAL before and after recall. n = 6–8/group. Data are representative of three independent experiments that yielded similar results. p < 0.05 compared with *naive, [†]OVA, and [‡]HDM mice, respectively.

inflammatory response to resolve completely (Figures 3B and 3C). After this period, mice were reexposed for 3 consecutive days to aerosolized OVA and killed approximately 72 hours after the last challenge (Figure 3A). We observed that mice that initially received either OVA or HDM alone showed no significant changes in the BAL cellular profile compared with naive mice after *in vivo* recall with OVA (Figures 3B and 3C). In contrast, OVA reexposure to HDM/OVA mice induced a significant increase in the total cell number compared with all other groups (Figure 3B). Moreover, this inflammatory response was characterized by a considerable accumulation of both mononuclear cells and eosinophils (Figures 3B and 3C).

Histologic evaluation of lung tissue. Histologic examination of the lung tissue confirmed the observations made in the BAL. Indeed, we observed no evidence of inflammation in the lungs of HDM/OVA and HDM mice that were killed just before *in vivo* recall with OVA, verifying that the lung inflammatory response had fully resolved over the 8-week rest period (data not shown). In addition, after *in vivo* recall with OVA, the lungs of OVA and HDM-alone mice resembled that of naive mice because there was no evidence of either peribronchial or perivascular inflammation (Figures 4A–4C). Moreover, no eosinophils could

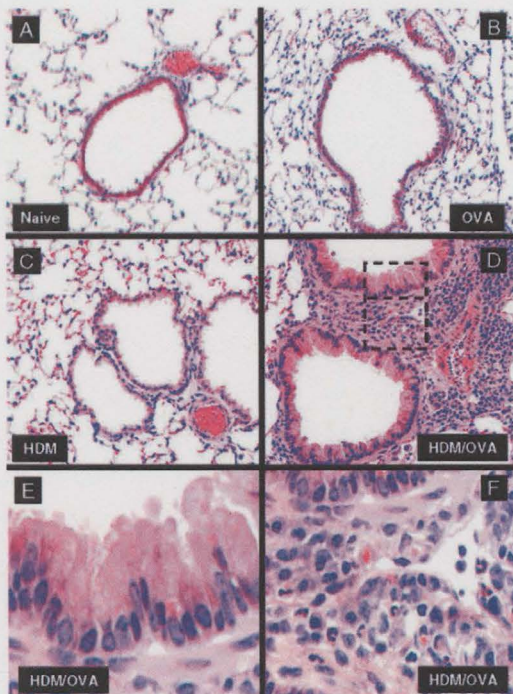


Figure 4. Light photomicrographs of paraffin-embedded sections of lung tissue obtained 72 hours after *in vivo* recall with OVA alone. (A) naive mice; (B) mice initially exposed to OVA; (C) mice initially exposed to HDM; (D) mice initially exposed to HDM concurrently with OVA (HDM/OVA); (E) magnified image of the upper dashed box shown in D, demonstrating goblet cell hyperplasia; (F) magnified image of the lower dashed box shown in D, demonstrating the presence of eosinophils among the inflammatory infiltrate. All panels show tissue stained with hematoxylin and eosin. Original magnification: A–D, $\times 150$; E and F, $\times 400$.

be found among the residing lung cells. In contrast, the lungs of HDM/OVA mice exhibited pronounced perivascular and peribronchial inflammation (Figure 4D), with clear evidence of goblet cell hyperplasia (Figure 4E). Furthermore, in agreement with the BAL data, the inflammatory infiltrate observed in the lungs of HDM/OVA mice after OVA recall was predominantly composed of mononuclear cells and eosinophils (Figure 4F).

Flow cytometric analysis of T1/ST2 expression on lung mononuclear cells. To further evaluate the composition of the lung mononuclear infiltrate, we performed flow cytometric analysis. Specifically, we wished to ascertain whether the increased influx of mononuclear cells after *in vivo* OVA recall in HDM/OVA mice was accompanied by an increase in the percentage of CD4⁺ T cells and, more specifically, in activated Th2 cells. After recall with OVA, we detected an approximate doubling in the percentage (10-fold increase in the number) of CD3⁺/CD4⁺ cells in the lung mononuclear cell population of HDM/OVA mice compared with naive and control groups (Figure 5). Moreover, this increase was associated with two- to threefold and four- to fivefold increases in the percentage of CD3⁺/CD4⁺ cells expressing CD69 (an early T-cell activation marker) or T1/ST2 (a putative marker of Th2 effector cells [18, 19]), respectively, over naive and control animals, and a five- to sixfold increase in the percentage of CD3⁺/CD4⁺ cells expressing both CD69 and T1/ST2.

Further evidence of OVA-specific immune-effector function. To extend our evaluation of OVA-specific immune-effector

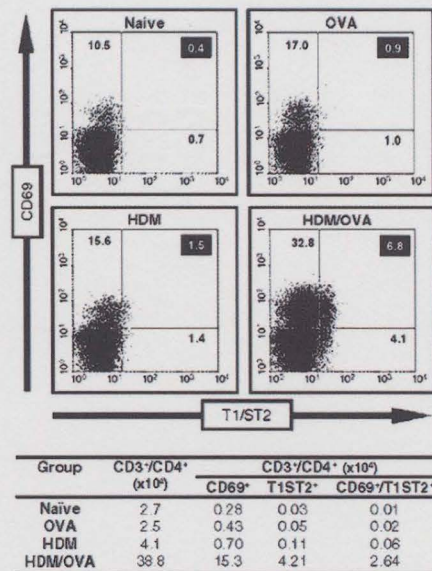


Figure 5. T1/ST2 and CD69 expression on CD4⁺ T lymphocytes obtained from the lungs of mice after *in vivo* recall with OVA. Lung mononuclear cell fraction was obtained by enzymatic digestion of whole lungs that were collected 72 hours after the last (third) OVA exposure. Lungs from 4–5 mice were pooled for each group. Cells were stained with mAbs against CD3, CD4, CD69, and T1/ST2 or with isotype controls and analyzed by four-color flow cytometry; more than 300,000 events were collected for each group. Dot plots shown are gated on CD3⁺/CD4⁺ cells. Percentages are shown in the top right corner of each quadrant while the table provides the absolute number of cells expressing the given markers.

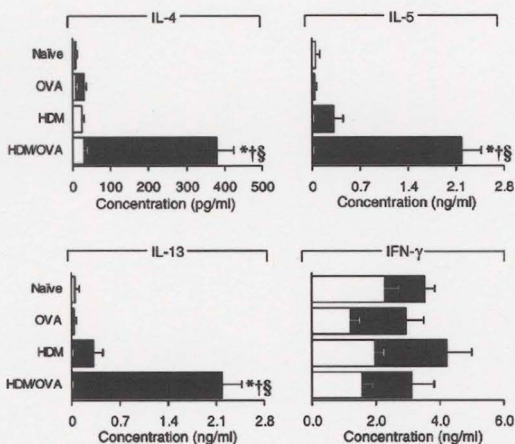


Figure 6. Th2-associated cytokine production after *in vitro* stimulation of splenocytes with OVA. Splenocytes were isolated from whole spleens collected 72 hours after OVA *in vivo* recall from OVA, HDM, and HDM/OVA mice. Splenocytes from individual mice were cultured and stimulated for 5 days with OVA (black bars) or medium alone (white bars), in triplicate. n = 8/group. Data are representative of three independent experiments that yielded similar results. p < 0.05 compared with *naive, †OVA, and §HDM mice, respectively.

function, we examined the cytokine profile in the supernatant of OVA-stimulated splenocyte cultures and the levels of OVA-specific immunoglobulins in the serum. We detected a considerable increase in the production of the Th2 cytokines IL-4, IL-5, and IL-13 from splenocytes obtained from HDM/OVA mice compared with naive and control groups (Figure 6). Furthermore, there were no significant differences in the levels of IFN-γ among all groups (Figure 6). In addition, we detected significantly increased levels of the OVA-specific Th2-associated immunoglobulins IgE and IgG₁ in the sera of HDM/OVA mice versus naive and control animals; no significant differences were observed in Th1-associated OVA-specific IgG_{2a} between OVA and HDM/OVA groups (Figure 7).

Impact of the lung inflammatory response on lung function.

Finally, we investigated the physiologic impact of the observed inflammatory response on airway function of HDM/OVA mice after OVA recall *in vivo*. We evaluated the respiratory resistance after increasing doses of methacholine in naive and HDM/OVA mice. HDM/OVA mice that were killed after the rest period but just before *in vivo* OVA recall (HDM/OVA before recall) demonstrated, as expected (7), a residual increase in airway resistance over naive animals (Figure 8). This is attributable to prolonged HDM exposure because HDM-alone mice demonstrated similar increases after the same period of cessation (data not shown and Reference 7). After *in vivo* recall with OVA (HDM/OVA after recall), we observed a marked increase in airway resistance, which was statistically significant at all doses of methacholine when compared with naive mice and HDM/OVA mice before recall.

DISCUSSION

There is increasing recognition of the concept that the immunologic milieu in which an antigen is first encountered largely determines the type of immune response that subsequently evolves.

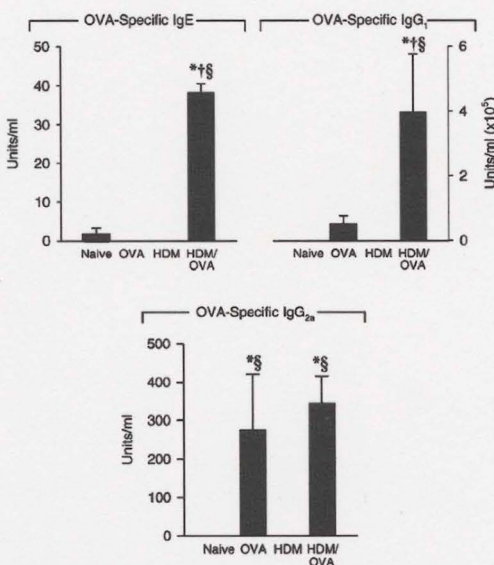


Figure 7. Th2-associated OVA-specific immunoglobulin levels after *in vivo* OVA recall. Serum was collected from naive mice (untreated) and OVA, HDM, and HDM/OVA mice after *in vivo* OVA recall, and the levels of OVA-specific IgE, IgG₁ (Th2-affiliated), and IgG_{2a} (Th1-affiliated) were measured. n = 4–5/group. Data are representative of three independent experiments that yielded similar results. p < 0.05 compared with *naive, †OVA, and §HDM mice, respectively.

In the context of allergic airway sensitization, a series of experimental studies provide supporting evidence for this concept. Indeed, although repeated exposure to aerosolized OVA alone resulted in inhalation tolerance (3–5), exposure in a granulocyte-macrophage colony-stimulating factor (GM-CSF)-enriched mi-

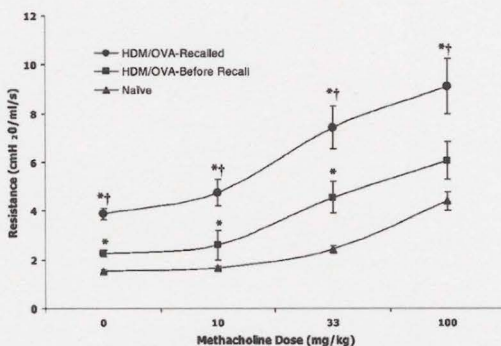


Figure 8. Assessment of airway hyperreactivity to methacholine after *in vivo* recall with OVA. Total respiratory resistance was measured at increasing doses of methacholine in naive mice, HDM/OVA mice that were killed after rest (~ 8 weeks) but before OVA *in vivo* recall and HDM/OVA mice killed after OVA *in vivo* recall. n = 8–9/group; p < 0.05 compared with *naive and †HDM/OVA (before recall) mice. Statistical analysis was performed using one-way ANOVA with Fisher’s LSD *post hoc* test.

croenvironment elicited the generation of an OVA-specific Th2 response (16), whereas conditioning with GM-CSF and IL-12 induced a Th1-type response (20). Although the airway microenvironment, in these studies, was altered by the overexpression of proinflammatory cytokines, the recent observation that intranasal administration of HDM extract elicits, without additional adjuvants, both acute (6) and chronic (7) airway inflammation intimates that HDM, a common environmental allergen, is intrinsically capable of creating an airway milieu conducive to allergic sensitization. Here, we investigated whether an HDM-conditioned lung microenvironment could subvert the expected immunologic response to aerosolized OVA.

We observed that a short exposure (10 days) to HDM alone or to HDM and OVA resulted in equivalent levels of airway inflammation. This was not surprising given that HDM alone elicits a robust inflammatory response in the lung. That we also observed an increase, albeit modest, in the production of OVA-specific Th2-associated cytokines by splenocytes harvested from HDM/OVA mice suggested that a degree of OVA-specific sensitization had occurred. In other words, short concurrent exposure may have been sufficient to subvert the development of OVA-specific inhalation tolerance.

Impelled by this observation, we explored whether extending the period of concurrent exposure to 5 weeks would strengthen the degree of OVA-specific sensitization. Indeed, production of OVA-specific Th2 cytokines by splenocytes harvested from mice concurrently exposed to HDM and OVA was considerably enhanced. In addition, we documented elevated serum levels of OVA-specific IgG₁ under these protracted exposure conditions. Of interest, this observation is in agreement with that of Gough and coworkers (10) who demonstrated an enhancement of OVA-specific IgE after concurrent intraperitoneal injection of the major dust mite allergen Der p 1 with OVA and with that of Deplazes and colleagues (9) who made a similar observation in dogs. Notably, the serum level of OVA-specific immunoglobulins was the only outcome evaluated in these studies. Nevertheless, our findings of increased levels of OVA-specific IgG₁ and Th2 cytokine production by OVA-stimulated splenocytes on extended concurrent exposure to HDM and OVA told us nothing about whether an inflammatory response would be triggered in the lung/airway compartment on subsequent exposure to OVA (21).

Thus, to evaluate the impact of extended concurrent allergen exposure on the development of OVA-specific memory more conclusively and, particularly, to ascertain whether this would result in airway inflammation on subsequent OVA exposure, mice were rested and then rechallenged, *in vivo*, with OVA. Our data demonstrate that *in vivo* recall with OVA led to a robust Th2-type airway inflammatory response only in mice that had initially been exposed to OVA in the context of an HDM-conditioned airway. Importantly, this inflammatory response was associated with significantly increased bronchial hyperreactivity. Furthermore, we also observed elevated serum levels of OVA-specific IgE and IgG₁, as well as Th2 cytokine production by splenocytes. Collectively, our data convincingly demonstrate that exposure to HDM subverts the expected immunologic outcome of passive OVA airway exposure (i.e., inhalation tolerance) and facilitates instead a response that immunologically and functionally resembled asthma.

Mechanistically, it has been proposed that HDM may contribute to allergic sensitization in a number of ways. For example, there is evidence that HDM disrupts cell monolayers and degrades tight junctions in the airway epithelium *in vitro* (22–25). This would facilitate its penetration across the airway epithelial barrier (26) and, presumably, increase its accessibility to antigen presenting cells located in the subepithelial compartment (26).

In addition, it has been suggested that HDM may privilege the generation of a Th2-polarized response by cleaving CD25 (IL-2R α chain [27]) and by modulating the balance between IL-4 and IFN- γ (28). It has also been suggested that cleavage of CD23 (low-affinity IgE receptor) may promote and enhance an IgE immune response (29, 30). Notably, these various effects are related to HDM's intrinsic proteolytic activity, an attribute shared by a number of common environmental aeroallergens (31–35) and fungi (11, 36, 37). In this regard, Kheradmand and colleagues (11) have recently shown that the proteolytic activity of *Aspergillus fumigatus* is necessary and sufficient to elicit airway eosinophilic inflammation. However, it has become increasingly clear that proteases can have more direct proinflammatory effects. Indeed, it has been shown that various dust mite allergens act on bronchial epithelial cells *in vitro* to elicit the production of a number of cytokines (38–40), including GM-CSF, a powerful maturation and activating signal for dendritic cells, and that such an effect is elaborated via proteolytic activity (39, 40). We suggest that HDM-induced GM-CSF may play a significant role in the findings that we report here. First, there is evidence that exogenous administration of GM-CSF to the airway generates a Th2 immune-inflammatory response not only to OVA (16) but also to allergens with weak proteolytic activity, such as ragweed (21). Moreover, we have recently shown that intranasal administration of HDM daily for 10 days induces allergic sensitization and Th2 airway inflammation, both of which are substantially reduced by concurrent treatment with anti-GM-CSF antibodies (6). Thus, we would propose that HDM may elicit a cascade that conditions the airway microenvironment with cytokines such as GM-CSF, and likely others, to facilitate the generation of a long-lived Th2-polarized immune-inflammatory response to OVA and itself. Unfortunately, a protocol that involves continuous exposure to HDM and OVA for 5 weeks disallowed the use of GM-CSF or GM-CSF receptor-deficient mice due to their short survival span; moreover, GM-CSF neutralization was prohibitive, economically, and ill-advised, immunologically, due to the likelihood of triggering a type III hypersensitivity reaction with such prolonged antibody treatment.

In summary, our data show that exposure to HDM extract establishes a mucosal environment that facilitates the generation of a bona fide Th2-polarized memory response to an otherwise innocuous antigen, OVA. We believe that the clinical significance of these findings is apparent. Indeed, it is likely that humans are simultaneously exposed to an assortment of different aeroallergens with different antigenic potential. From this perspective, exposure to the pervasive aeroallergen HDM may facilitate or amplify the response to other allergens with a lower intrinsic ability to cause disease. By extrapolation, our data may highlight the importance of controlling exposure to HDM, and other indoor allergens, in the overall management of asthma and specifically to curtail the development of poli-allergen sensitization. Moreover, the implications of concurrent exposure can probably be extended beyond the domain of aeroallergens. For example, the potential of certain pollutants to elicit allergic airway disease has been established in both experimental models (41, 42) and in humans (43), and perhaps the ability of certain viruses, such as respiratory syncytial virus (RSV), to facilitate allergic airway disease stems from a similar mechanism (44). Ultimately, the findings that we report here compel us to consider that whether a certain aeroallergen will generate allergic airway disease may depend, at least in part, not on simply being exposed to it but rather the context in which that exposure occurs.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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**House Dust Mite Facilitates Ovalbumin-specific Allergic
Sensitization and Airways Inflammation**

Ramzi Fattouh, Mahmoud A. Pouladi, David Alvarez, Jill R. Johnson, Tina D. Walker,
Susanna Goncharova, Mark D. Inman and Manel Jordana

Online Supplement

Materials and Methods

COLLECTION AND MEASUREMENT OF SPECIMENS:

72 hours after the last challenge mice were killed and bronchoalveolar lavage (BAL) fluid, lungs, blood, and spleen were collected. BAL was performed as previously described (1). Briefly, lungs were dissected and the trachea was cannulated with a polyethylene tube (outer/inner diameter = 0.965/0.58mm; Becton Dickinson, Sparks, MD, USA). Lungs were lavaged twice with PBS (0.25ml followed by 0.2ml) and approximately 0.3ml of the instilled fluid was consistently retrieved. Total cell counts were then determined using a hemocytometer. Each BAL sample was then centrifuged and the supernatants collected and stored at -20°C. Cell pellets were subsequently resuspended in PBS and smears were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, PA, USA) at 300rpm for 2min. Protocol Hema 3 stain set (Fisher Scientific, Toronto, ON, Canada) was used to stain all smears. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Where applicable, following BAL, lungs were inflated with 10% formalin at a constant pressure of 20cm H₂O, and then fixed in 10% formalin for at least 24 hours. Tissues were then embedded in paraffin and 3-µm-thick sections were cut and stained with hematoxylin and eosin. Peripheral blood (PB) was collected by retroorbital bleeding and serum was obtained by centrifugation of whole blood after 30min incubation at 37°C and stored at -20°C.

SPLENOCYTE CULTURE:

Harvested spleens were placed in sterile tubes containing sterile HBSS. Splenocytes were isolated by triturating whole spleens through a 40µm cell strainer (Becton Dickinson)

using the plunger from a 5ml syringe into HBSS. Following centrifugation at 1200rpm for 10min at 4°C, red blood cells were lysed using ACK lysis buffer (0.5 M NH₄Cl, 10 mM KHCO₃, and 0.1 nM Na₂EDTA, pH 7.2-7.4). Splenocytes were then washed once with HBSS and then again with RPMI supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen, Grand Island, NY, USA), and 0.1% β-mercaptoethanol (Invitrogen). Splenocytes were then resuspended in complete RPMI at a concentration of 8x10⁶ cells/ml and cultured in medium alone, or with medium supplemented with OVA (40µg/ml) in a flat-bottom, 96-well plate (Becton Dickinson), in triplicate; 100µl of cell suspension + 100µl of medium-OVA per well. After 5 d of culture, supernatants were harvested and triplicates were pooled for cytokine measurement.

CYTOKINE AND IMMUNOGLOBULIN MEASUREMENTS:

ELISA kits for IL-4, -5, -13, and IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA). Each of these assays has a threshold of detection between 1.5-5pg/ml. Levels of OVA-specific IgE in the serum were measured using a previously described antigen-capture ELISA method (1), while OVA-specific serum IgG1 and IgG2a were measured by sandwich ELISA with OVA in the solid phase, as previously described in detail in (2). Quantity of OVA-specific Igs were determined relative to in-house standardized serum, obtained either from mice sensitized to OVA through a conventional i.p. sensitization model for IgE and IgG₁ standards (1) or according to our Th1-polarized mucosal sensitization model for IgG_{2a} standard (3). As such, sample Ig levels are expressed as Units/ml relative to the above described standard mouse sera.

LUNG CELL ISOLATION AND FLOW CYTOMETRIC ANALYSIS OF LUNG MONONUCLEAR CELLS:

Lung cells were isolated as previously described with slight modifications (4). Briefly, total lung mononuclear cells were isolated by collagenase digestion (Collagenase type III; Life Technologies Inc., Burlington, ON, Canada) followed by discontinuing gradient centrifugation in 30% and 60% Percoll (Pharmacia Biotech AB, Uppsala, Sweden). The interface containing the mononuclear cells was collected, washed twice with PBS, and stained with a panel of antibodies. To minimize nonspecific binding cells were first preincubated with FcBlock (anti-CD16/CD32; PharMingen, Mississauga, ON, Canada). For each antibody combination, $0.5-1 \times 10^6$ cells were incubated with mAbs at 4°C for 30min. Cells were then washed and fixed in 1% paraformaldehyde. Data were collected using a FACScan™ (Becton Dickinson) four-colour flow cytometer and analyzed using WinMDI software (Scripps Research Institute, La Jolla, CA, USA). The following antibodies were used: anti-CD3 (Cy-Chrome-conjugated 145-2C11), anti-CD4 (allophycocyanin-conjugated RM4-5), and anti-CD69 (phycoerythrin-conjugated H1-2F3), all purchased from BD PharMingen; anti-T1/ST2 (3E10) was provided by Millennium Pharmaceuticals Inc. (Cambridge, MA, USA), and fluorescein isothiocyanate-labeled in-house according to a standard protocol (5). All appropriate isotype controls were used (BD PharMingen). Antibodies were titrated to determine optimal concentration.

AIRWAY HYPERREACTIVITY MEASUREMENTS:

Airway responsiveness was measured on the basis of the response of total respiratory system resistance to increasing i.v. (internal jugular vein) doses of methacholine (MCh) as previously described (6). Evaluation of airway responsiveness was based on the peak

respiratory resistance measured in the 30s after the saline and MCh challenges. Briefly, mice were anesthetized with tribromoethanol (287 mg/kg intraperitoneally), prepared according to a standard protocol (7). The trachea was exposed and cannulated, and a constant inspiratory flow was delivered by mechanical ventilation (RV5; Voltek Enterprises Inc., Toronto, Ontario, Canada). Heart rate and oxygen saturation were monitored via infrared pulse oximetry (Biox 3700; Ohmeda, Boulder, Colorado, USA), using a standard ear probe placed over the proximal portion of the mouse's hind limb. Paralysis was achieved using pancuronium (0.03 mg/kg intravenously) to prevent respiratory effort during measurement. Respiratory resistance was measured after consecutive intravenous injections of saline, followed by 10, 33, and 100 µg/kg of MCh (ACIC [Can], Brantford, Ontario, Canada), each delivered as a 0.2-mL bolus. During each MCh dosing, the mouth-pressure signal from the ventilator was converted to a digital signal (Dash 16; Metrabyte, Staughton, Massachusetts, USA) and recorded at 400 Hz on a PC. Respiratory resistance was calculated as described previously (6).

DATA ANALYSIS:

Data are expressed as mean ± SEM. Results were interpreted using ANOVA with a Tukey post-hoc test, unless otherwise indicated. Differences were considered statistically significant when $P < 0.05$.

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— Chapter 3 —

Transforming Growth Factor- β Regulates House Dust Mite-Induced Allergic Airway Inflammation but Not Airway Remodeling

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See *Preface* for details regarding authorship.

Summary and Central Message: This article is the first of two that investigate the mechanisms by which chronic allergic pulmonary inflammation may promote the development of airway remodeling. Specifically, we seek to elucidate the importance of the preeminent TGF- β – Eosinophil pathway in this process. In this study, we examine the role of TGF- β in the generation of HDM-induced remodeling. We found that impairment of TGF- β function, in either a continuous or intermittent HDM-exposure model, did not affect the development of remodeling. Moreover, we observed that interfering with TGF- β activity potentiated systemic Th2 immune responses (HDM-specific IgG₁ and IgE and splenocyte cytokine production) and exacerbated airway inflammation. Importantly, TGF- β blockade also enhanced AHR. Collectively, these findings demonstrate that TGF- β is not absolutely required for remodeling to develop in the context of HDM exposure and suggest that TGF- β functions primarily as a negative regulator of allergic immune-inflammatory responses. Given that OVA-induced remodeling is dependent on the activity of TGF- β , our data further intimate that the molecular regulation of airway remodeling may be dependent on the nature of the allergen itself.

Transforming Growth Factor- β Regulates House Dust Mite-induced Allergic Airway Inflammation but Not Airway Remodeling

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Rationale: It is now believed that both chronic airway inflammation and remodeling contribute significantly to airway dysfunction and clinical symptoms in allergic asthma. Transforming growth factor (TGF)- β is a powerful regulator of both the tissue repair and inflammatory responses, and numerous experimental and clinical studies suggest that it may play an integral role in the pathogenesis of asthma.

Objectives: We investigated the role of TGF- β in the regulation of allergic airway inflammation and remodeling using a mouse model of house dust mite (HDM)-induced chronic allergic airway disease.

Methods: We have previously shown that intranasal administration of an HDM extract (5 d/wk for 5 wk) elicits robust Th2-polarized airway inflammation and remodeling that is associated with increased airway hyperreactivity. Here, Balb/c mice were similarly exposed to HDM and concurrently treated with a pan-specific TGF- β neutralizing antibody.

Measurements and Main Results: We observed that anti-TGF- β treatment in the context of either continuous or intermittent HDM exposure had no effect on the development of HDM-induced airway remodeling. To further confirm these findings, we also subjected SMAD3 knockout mice to 5 weeks of HDM and observed that knockout mice developed airway remodeling to the same extent as HDM-exposed littermate controls. Notably, TGF- β neutralization exacerbated the eosinophilic infiltrate and led to increased airway hyperreactivity.

Conclusions: Collectively, these data suggest that TGF- β regulates HDM-induced chronic airway inflammation but not remodeling, and furthermore, caution against the use of therapeutic strategies aimed at interfering with TGF- β activity in the treatment of this disease.

Keywords: immunology; allergic asthma; mouse model; lung

Asthma is a chronic disease that is characterized clinically by variable airflow obstruction and a decline in airway function (1). It is now believed that these outcomes are the consequences of two distinct, although likely connected, processes—namely,

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Transforming growth factor (TGF)- β appears to play a prominent role in the development of airway remodeling, a recognized feature of chronic allergic asthma.

What This Study Adds to the Field

This research suggests that airway remodeling may be independent of TGF- β , and that therapeutic strategies to prevent remodeling through TGF- β interference may have detrimental effects by increasing eosinophilic infiltration and airway hyperreactivity.

allergic airway inflammation and airway remodeling (2–5). Experimental studies in animal models of allergic airway disease have afforded considerable understanding with respect to the nature and mechanisms that drive allergic inflammation; however, relatively speaking, our knowledge of the mechanistic processes that underlie the development of airway remodeling remains limited and in some cases controversial. Moreover, although it is generally accepted that inflammation-associated damage consequently triggers tissue reparative responses as a means of restoring tissue integrity, whether and how this is coordinately regulated in allergic airway disease are also poorly understood.

In the context of allergic asthma, the pathologic hallmarks of airway remodeling include increased subepithelial extracellular matrix deposition, alterations in the airway epithelium (goblet cell hyperplasia) leading to excessive mucus production, and airway smooth muscle thickening resulting from smooth muscle cell hypertrophy and hyperplasia (6, 7). Among the various factors that are believed to be involved, transforming growth factor (TGF)- β , a powerful regulator of the tissue repair response, has been strongly implicated in the development of airway remodeling. Clinical studies have documented increased TGF- β expression on both the level of mRNA and protein in bronchial biopsies and in the bronchoalveolar lavage (BAL) of humans with asthma compared with control subjects (8–12). Similarly, TGF- β expression has also been shown to be increased in animal models of allergic airway disease (13–17). In addition, the number of TGF- β -expressing epithelial or submucosal cells has been correlated with the basement membrane thickness in patients with asthma (12). Direct evidence in support of a role for TGF- β in the development of allergic airway remodeling comes from a study by McMillan and colleagues, who showed that TGF- β blockade using a neutralizing antibody (Ab) reduced the extent of ovalbumin (OVA)-induced remodel-

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eling in a chronic mouse model (18). In addition, two very recent studies, one using SMAD3 knockout (KO) animals (19) and the other using blocking Abs (20), have also demonstrated that TGF- β contributes to the development of airway remodeling in similar OVA-based systems. Animal studies demonstrating a critical role for TGF- β in the pathogenesis of related diseases involving aberrant tissue repair, such as liver, kidney, and lung fibrosis, further support the notion of TGF- β involvement in allergic airway remodeling (21–24).

Importantly, it is clear that TGF- β is also one of the most potent negative regulators of inflammation (25). Indeed, TGF- β -deficient mice die soon after birth due to rampant, multifocal inflammation (26). This activity may also be of particular relevance to allergic airway disease as TGF- β is believed to contribute, at least in part, to the regulation of the Th2-polarized airway inflammatory response that is characteristic of this disease. Numerous *in vitro* studies have collectively shown that TGF- β suppresses the activity and/or proliferation of various inflammatory cells, including Th2 cells, B cells, macrophages, and eosinophils (25, 27–29), and moreover, TGF- β can promote their apoptosis (30–32). Three separate studies have investigated the impact of genetically interfering with TGF- β activity on allergic sensitization and airway inflammation in acute models of allergic airway disease using OVA. Their findings consistently demonstrate that abrogation of TGF- β or TGF- β signaling pathways substantially potentiates the immune response and exacerbates the allergic inflammatory response (33–35).

In light of these reports, we and others have proposed that TGF- β may play a dual role in asthma by coordinately regulating both the inflammatory and reparative responses; specifically, that TGF- β may dampen the inflammatory response elicited upon aeroallergen exposure in a sensitized individual while subsequently initiating tissue repair processes. In this study, we investigated the role of TGF- β in the regulation of chronic allergic airway inflammation and remodeling. To address this issue experimentally, we conducted a series of loss-of-function experiments in a model of chronic allergic airway disease induced by respiratory mucosal exposure to a clinically relevant aeroallergen, house dust mite (HDM). We have previously shown that repeated exposure to an HDM extract elicits robust Th2-polarized airway inflammation and remodeling that are associated with increased bronchial hyperreactivity (36). Here we report that mice exposed to HDM and concurrently treated with a pan-neutralizing anti-TGF- β (α TGF- β) Ab developed airway remodeling comparable to mice exposed to HDM and treated with an irrelevant Ab control. Similarly, we also observed that HDM-exposed SMAD3 KO mice developed remodeling to the same extent as HDM-exposed wild-type (WT) littermate control animals. Interestingly, we further demonstrate that TGF- β neutralization exacerbates the eosinophilic inflammatory infiltrate and that this was associated with a significant increase in bronchial hyperreactivity. Collectively, these data suggest that TGF- β regulates HDM-induced airway inflammation, but that it may not play a critical role in the generation of HDM-induced airway remodeling.

Some of the results of these studies have been previously reported in the form of an abstract (37).

METHODS

Animals

Female Balb/c mice (6–8 wk old) were purchased from Charles River Laboratories (Ottawa, ON, Canada). SMAD3 heterozygous mice (129SV/EV \times C57B/6 background; courtesy of Dr. A. Roberts, National Institutes of Health, Bethesda, MD) (38) were bred in-house.

The genotypes of both WT littermate and SMAD3 KO mice were determined by polymerase chain reaction analysis on tail DNA obtained from 3-week-old animals. All mice were housed under specific pathogen-free conditions and maintained on a 12-hour light:dark cycle, with food and water *ad libitum*. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada).

Sensitization Protocols

Allergen administration. HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in sterile saline at a concentration of 2.5 mg (protein)/ml and 10 μ l were administered to isoflurane-anesthetized Balb/c mice intranasally. SMAD3 KO and littermate control mice received the same amount of HDM per dose (i.e., 25 μ g) delivered in 15 μ l of saline.

Ab administration. Murine pan-neutralizing α TGF- β specific for all forms of murine TGF- β or an irrelevant murine isotype-matched control Ab (IgG) were administered to anesthetized mice intraperitoneally at a dose of 10 or 100 μ g (corresponding to \sim 0.5 and 5 mg/kg, respectively) in 0.5 ml of sterile phosphate-buffered saline immediately before HDM administration.

Continuous HDM exposure protocol. Separate groups of mice were exposed daily to HDM or saline for 5 consecutive days a week followed by 2 days of rest for a total of 5 weeks. Depending on the experiment, α TGF- β or control Ab was concurrently administered either at the beginning of the third week (Day 14) or on the fourth day of HDM exposure (Figures 1A and 1B). Ab was administered every other day until the mice were killed, 72 hours after the last challenge.

Intermittent HDM exposure protocol. Separate groups of mice were exposed daily to HDM or saline for 10 consecutive days followed by approximately 30 days of rest. We have previously shown that 10 days of HDM exposure elicits robust Th2-polarized allergic sensitization and airway inflammation (39). Saline- and HDM-sensitized mice were subsequently subjected to six cycles of saline or HDM reexposure, respectively; each cycle consisted of daily intranasal exposure for 3 consecutive days followed by 12 days of rest. In addition, mice were concurrently treated every other day with α TGF- β or control IgG over each of the six reexposure cycles beginning 2 days before the first saline/HDM exposure and ending on the second day after the last (i.e., third) saline/HDM exposure, for a total of four Ab administrations per cycle. Mice were killed 72 hours after the last challenge (Figure 1C).

Collection and Measurement of Specimens

BAL fluid, lungs, blood, and spleen were collected at the time of killing. BAL was performed as previously described (40). Total cell counts were determined using a hemocytometer. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytologic criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Where applicable, after BAL, the right lung was dissected for tissue homogenate preparation; the left lung was inflated with 10% formalin at a constant pressure of 20 cm H₂O, and then fixed in 10% formalin for 48 to 72 hours. Peripheral blood was collected by retro-orbital bleeding and serum was obtained and stored at -20° C. Harvested spleens were placed in sterile tubes containing sterile Hanks' balanced salt solution. See the online supplement for additional details on the methods used to make these measurements.

Preparation of Lung Tissue Homogenate and Hydroxyproline Measurement

See the online supplement for details on the methods used to make these measurements.

Histology and Immunohistochemistry

After formalin fixation, tissues were embedded in paraffin, and 3- μ m-thick cross-sections of the left lung were cut and stained with hematoxylin and eosin, Picro Sirius red (PSR), and periodic acid-Schiff (PAS). Immunohistochemistry for α -smooth muscle actin (α -SMA) was also performed. See the online supplement for additional details on the methods used to make these measurements.

Morphometric Analysis

Images for morphometric analysis were captured with OpenLab software (version 3.0.3; Improvision, Guelph, ON, Canada) via a Leica

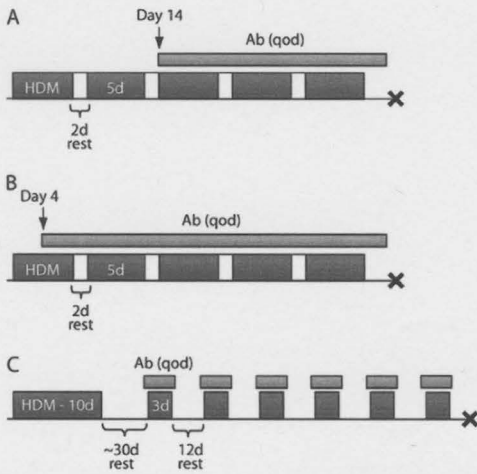


Figure 1. Schematic diagrams of the experimental protocols used. (A) Continuous house dust mite (HDM) exposure protocol: Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) during the last 3 weeks of exposure (i.e., starting on Day 14) with either a pan-neutralizing anti-transforming growth factor- β (α TGF- β ; 10 or 100 μ g/dose) antibody (Ab), or the control IgG Ab (100 μ g/dose). (B) Continuous HDM exposure protocol with extended treatment: Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) beginning at Day 4 of exposure with either α TGF- β (100 μ g/dose) or the control IgG Ab (100 μ g/dose). (C) Intermittent HDM exposure protocol: Separate groups of mice were exposed to saline or HDM for a period of 10 consecutive days and then rested for approximately 30 days. Mice were subsequently subjected to six cycles of saline or HDM reexposure; each cycle consisted of daily exposure for 3 consecutive days followed by 12 days of rest. In addition, mice were concurrently treated (every other day) with either α TGF- β (100 μ g/dose) or the control IgG Ab (100 μ g/dose), over each 3-day reexposure to saline or HDM as described in METHODS. In all protocols, mice were killed 72 hours after the last challenge unless otherwise stated.

camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada). Image analysis was performed using a custom computerized analysis system (Northern Eclipse software version 5; Empix Imaging, Mississauga, ON, Canada). Analysis of sections stained for α -SMA, PSR, and PAS were performed as previously described (41). See the online supplement for additional details on the methods used to make these measurements.

Splenocyte Culture

Splenocytes were isolated, resuspended in complete RPMI (Roswell Park Memorial Institute) at a concentration of 8×10^6 cells/ml and cultured in medium alone, or with medium supplemented with HDM (31.25 μ g/ml) in a flat-bottom, 96-well plate (Becton Dickinson, Mississauga, ON, Canada), in triplicate. After 5 days of culture, supernatants were harvested and triplicates were pooled for cytokine measurements. See the online supplement for additional details on the methods used to make these measurements.

Bioassay for TGF- β

See the online supplement for details on the methods used to make these measurements.

Cytokine and Immunoglobulin Measurements by ELISA

Levels of IL-4, IL-5, IL-13, and active TGF- β 1 were measured by ELISA using DuoSet kits purchased from R&D Systems (Minneapolis,

MN) according to the manufacturer's instructions. Levels of α TGF- β murine monoclonal Ab and HDM-specific IgE and IgG₁ were measured by sandwich ELISA. See the online supplement for additional details on the methods used to make these measurements.

Airway Responsiveness Measurements

Airway responsiveness was measured on the basis of the response of total respiratory system resistance to increasing intravenous (internal jugular vein) doses of methacholine as previously described (42, 43). See the online supplement for additional details on the methods used to make these measurements.

Data Analysis

Data were analyzed using SigmaStat version 2.03 (SPSS, Inc., Chicago, IL). Data are expressed as mean \pm SEM. Results were interpreted using analysis of variance with Fisher's least significant difference post hoc test, unless otherwise indicated. Differences were considered statistically significant when *P* values were less than 0.05.

RESULTS

Kinetic Analysis of HDM-induced Responses in the Lung

Consistent with our previous report, we observed that Balb/c mice exposed via the respiratory mucosa to HDM for 5 weeks develop robust Th2-polarized airway inflammation (36). Kinetic analysis of the BAL revealed that an increase in the total cell number is evident after just 1 week of HDM, peaks at 3 weeks, and is maintained at this level throughout the course of allergen exposure (Figure 2A). Furthermore, we observed that this process is associated with a progressive airway reparative response that results in altered airway structure (Figure 2A and Reference 36). These structural changes are incipient after 3 weeks of HDM exposure and overt after 5 weeks, and include increased subepithelial collagen deposition and smooth muscle thickening. The concept that inflammation and repair are related strongly implicates TGF- β . Thus, we examined the expression of TGF- β in the BAL of mice exposed to 1, 3, or 5 weeks of HDM. We observed that the concentration of active TGF- β 1 was substantially elevated after 3 and 5 weeks of HDM compared with untreated (0 wk) and saline exposed control animals (data not shown), and appeared to be increased after just 1 week of HDM (Figure 2B). Interestingly, we noted that the increase in active TGF- β 1 at 3 and 5 weeks corresponded with both the plateau in inflammation and the airway remodeling response. These findings supported our hypothesis that TGF- β may be dampening the chronic allergic inflammatory response while at the same initiating reparative processes.

To directly investigate this *in vivo* we used a pan-neutralizing α TGF- β Ab that is specific for the active form of all three mammalian TGF- β isoforms. This Ab has been previously shown to reduce the extent of OVA-induced allergic airway remodeling (18). Although the characteristics and functional properties of this Ab have been extensively assessed, we reconfirmed the functionality of each batch of Ab by testing its ability to block TGF- β 1 detection by ELISA (Figure 2C) and TGF- β -induced luciferase expression in the plasminogen activator inhibitor-1 luciferase-based (PAI-L) bioassay (data not shown). To determine whether the Ab is able to reach the airway walls and subepithelial space, we also tracked its kinetics and distribution *in vivo*, in three separate compartments: the serum, lung tissue, and airway lumen. Separate groups of mice were given a single intraperitoneal administration of α TGF- β and then killed 2, 6, and 12 hours postinjection. We observed that the α TGF- β Ab was already present at considerable levels in the serum and lung tissue 2 hours postinjection, could be detected in the airway lumen in as little as 6 hours, and that

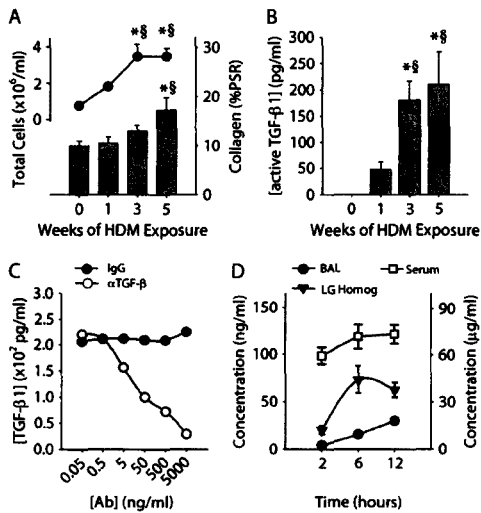


Figure 2. Kinetic analysis of house dust mite (HDM)-induced responses and pan-neutralizing anti-transforming growth factor- β (α TGF- β) antibody (Ab) distribution. (A) Kinetic analysis of HDM-induced airway inflammation and remodeling showing the total cell number in the bronchoalveolar lavage (BAL) (solid circles) and the extent of collagen deposition (shaded bars) based on morphometric analysis of Picro Sirius red-stained lung sections from mice exposed to 0 (naïve), 1, 3, or 5 weeks of HDM. (B) Concentration of active TGF- β 1 in the BAL of mice exposed to HDM for 0 (naïve), 1, 3, or 5 weeks. (C) Functionality of α TGF- β Ab. A fixed amount of active TGF- β 1 (200 pg/ml) was incubated with varying amounts of α TGF- β (open circles) or the isotype-matched control Ab (IgG; solid circles) for 2 hours and the amount of active TGF- β 1 was then measured by commercial ELISA. (D) Concentration of α TGF- β Ab in the BAL (solid circles), lung homogenate (LG Homog; solid triangles), and serum (open squares) of mice killed at 2, 6, and 12 hours after one 100- μ g injection. Where applicable, $n = 3$ –5/group. Data are expressed as mean \pm SEM. $P < 0.05$ compared with *0- and †1- week HDM-exposed groups, respectively.

concentrations steadily increased or were maintained thereafter (Figure 2D). This demonstrated that α TGF- β was indeed able to reach critical target regions in the lung.

Impact of TGF- β Blockade on the Development of HDM-induced Allergic Airway Remodeling

To investigate the role of TGF- β in the development of allergic airway remodeling, we exposed Balb/c mice to daily administrations of HDM (5 d/wk) for 5 weeks (continuous HDM exposure protocol) and concurrently treated them with 10 μ g (\sim 0.5 mg/kg) of α TGF- β Ab every other day during the last 3 weeks of exposure; this dose was shown to be efficacious in a previous study (18). Our intent was to begin TGF- β neutralization when levels of active TGF- β 1 in the BAL are low (Figure 2B) and airway remodeling is not yet apparent (Figure 2A), but at a time when allergic sensitization is fully established. We evaluated the impact of TGF- β blockade on the development of HDM-induced airway remodeling both qualitatively and by quantitative morphometric analysis of three prominent remodeling-associated events: subepithelial collagen deposition, smooth muscle thickness, and mucus production (as a marker of goblet cell hyperplasia). We observed significant increases in each of the three above parameters in HDM-exposed animals,

regardless of whether they were treated with α TGF- β or the control IgG, compared with saline-exposed control animals (Figure 3). In fact, treatment with α TGF- β had absolutely no impact on the development of HDM-induced remodeling, because no differences were observed between HDM-exposed α TGF- β -treated and HDM-exposed IgG-treated mice (Figure 3). Saline- and HDM-alone-exposed groups (i.e., receiving no Ab treatment) did not differ from saline-exposed α TGF- β -treated and HDM-exposed IgG-treated control animals, respectively (data not shown). That α TGF- β treatment had no effect on the remodeling response is unlikely to be a consequence of inadequate dosing because HDM-exposed mice treated with a 10-fold higher dose of α TGF- β Ab (100 μ g/dose every other day corresponding to \sim 5 mg/kg) displayed increases in collagen deposition, mucus production, and smooth muscle thickening that were virtually identical to HDM-exposed IgG-treated control animals (Figure 3). To confirm that TGF- β was being neutralized under these experimental conditions, we assessed the levels of bioactive TGF- β in the BAL fluid using the PAI/L bioassay for TGF- β . We observed that α TGF- β treatment decreased the levels of bioactive TGF- β in the BAL of HDM-exposed animals by approximately 80% compared with the HDM-exposed IgG-treated control group (Figure E1 of the online supplement), demonstrating that TGF- β was being effectively neutralized in our system.

We proceeded to examine whether TGF- β blockade was ineffective in the above experimental setting due to the timing of treatment initiation. Given that the levels of active TGF- β 1 are increased early on during HDM exposure (1–2 wk; Figure 2B), albeit to a lesser extent than at later time points, we questioned whether this early expression may have been sufficient to trigger a cascade of events that subsequently drove the remodeling response in a manner that was then independent of TGF- β . To address this, we subjected mice to the continuous HDM exposure protocol and treated them with α TGF- β or the control IgG over nearly the entire course of exposure. Even under this extended treatment regimen and using the 100- μ g dose, we observed that HDM-exposed α TGF- β -treated mice developed equivalent increases in subepithelial collagen, mucus production, and smooth muscle thickening as HDM-exposed IgG-treated mice (Figure 4). The similarity in the extent of collagen accumulation between HDM-exposed α TGF- β - and IgG-treated groups was further confirmed by a hydroxyproline assay (Figure E2).

Next, we ascertained whether the inability of TGF- β neutralization to inhibit airway remodeling was due to the severity of the inflammatory response that is elicited by this protocol. To this end, we developed a model of intermittent HDM exposure that was based on short, recurrent HDM rechallenges separated by 12-day rest periods (Figure 1C), rather than continuous HDM exposure. In this model, we observed that each 3-day HDM rechallenge elicited an eosinophilic inflammatory response, which then partially resolved over the subsequent rest period; total cells and eosinophils were decreased by approximately 60 and 65%, respectively, before subsequent rechallenge. Thus, the structural alterations that develop in this experimental protocol are associated with a less severe inflammatory response. We subjected mice to this intermittent exposure protocol and treated them with four 100- μ g administrations of α TGF- β or the IgG control Ab during each HDM rechallenge (Figure 1C). We documented that α TGF- β -treated HDM-exposed mice developed identical increases in subepithelial collagen deposition as HDM-exposed IgG-treated control animals (Figures 5A–5D), demonstrating that, even under these less severe conditions, TGF- β blockade had no effect on the development of HDM-induced airway remodeling.

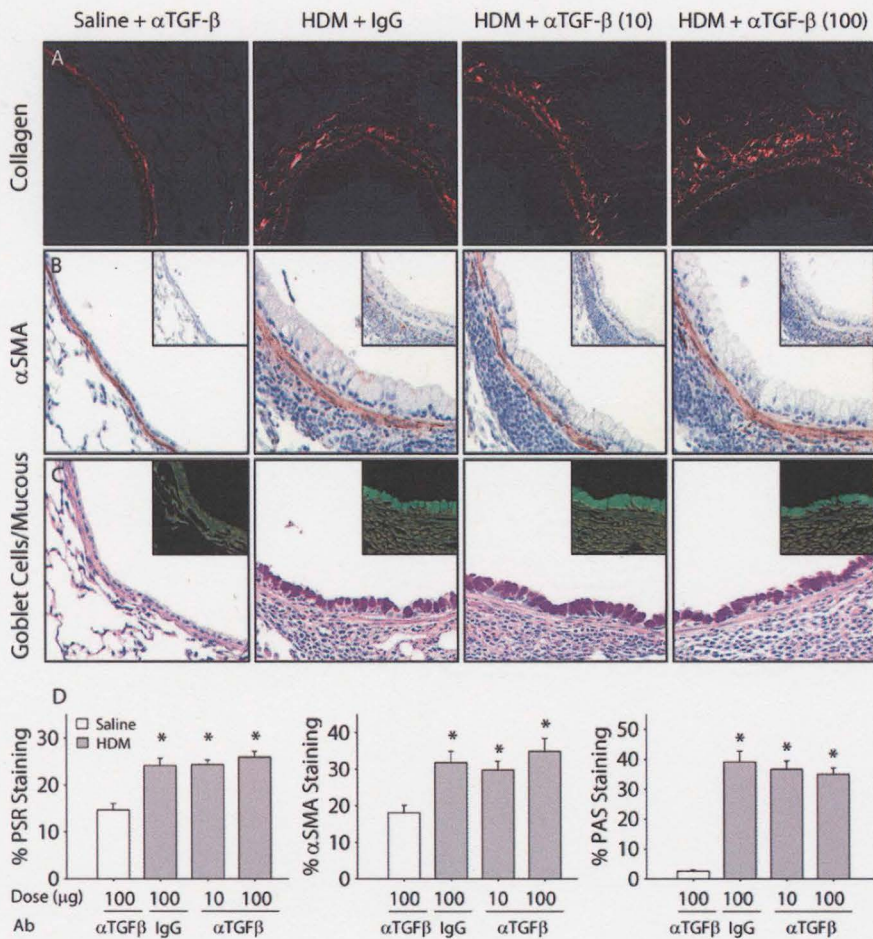


Figure 3. Impact of transforming growth factor (TGF)- β blockade on the development of house dust mite (HDM)-induced airway remodeling. Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) during the last 3 weeks of exposure with either anti-TGF- β (α TGF β) (10 or 100 μ g/dose) or the control IgG (100 μ g/dose) (Ab). Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition; (B) immunohistochemistry for α -smooth muscle actin (α -SMA) indicating contractile elements in the airway wall (brown; insets show non-specific staining in the corresponding negative control section); and (C) periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells (magenta; insets show color inverted image used for morphometric analysis). (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR, α -SMA, or PAS. All pictures were taken at $\times 20$ original magnification except insets in (C), which were at $\times 40$; $n = 5-7$ /group. Data are expressed

as mean \pm SEM and are from one of three independent experiments that yielded similar results. * $P < 0.05$ compared with the saline + α TGF- β -treated group. Ab = antibody.

Latent TGF- β protein is embedded throughout the extracellular matrix of most organs, especially the lungs, and it is believed that, once activated, TGF- β acts rapidly on only those cells that are in direct contact. Thus, we questioned whether such a mechanism would allow at least some TGF- β to escape neutralization by the blocking Ab. Importantly, we note that, in other experimental models of fibrosis, in which such a mechanism is believed to mediate fibrogenesis (44), treatment with the α TGF- β Ab used here significantly abrogated the development of fibrosis (Reference 45 and S. Lonning, personal communication). To confirm our results given this possibility, we used a genetically based approach to interfere with TGF- β activity. The use of TGF- β -deficient mice, although ideal, is effectively impossible given their extremely short life spans. As an alternative, we used SMAD3 KO mice because SMAD3 is a signaling molecule that has been shown to be a critical mediator of TGF- β -induced remodeling-associated gene expression (46). We subjected SMAD3 KO mice and their WT littermate controls to the continuous exposure protocol. After 5 weeks of HDM administration, we evaluated the development of airway remodeling in these mice. Morphometric analysis showed signif-

icant increases in collagen deposition (Figure 6A) and smooth muscle thickening (Figure 6B) in both WT and KO animals exposed to HDM compared with their respective saline-treated controls. Moreover, we found that the structural alterations observed in HDM-exposed SMAD3 KO mice were equivalent in magnitude to HDM-exposed WT control animals (Figure 6).

Impact of TGF- β Blockade on the Regulation of Chronic Allergic Airway Inflammation Induced by HDM

Given that TGF- β is a potent negative regulator of inflammation and has been shown to inhibit the activity, differentiation, and proliferation of various Th2-associated inflammatory cell types *in vitro*, we explored whether TGF- β blockade had any impact on the regulation of established chronic allergic airway inflammation. We exposed mice to the continuous HDM protocol and blocked TGF- β during the last 3 weeks of exposure using the neutralizing α TGF- β Ab. Treatment was initiated at this time to allow sensitization and the inflammatory response to become fully established. Analysis of the cellular infiltrate in the BAL revealed that mice exposed to HDM and treated with

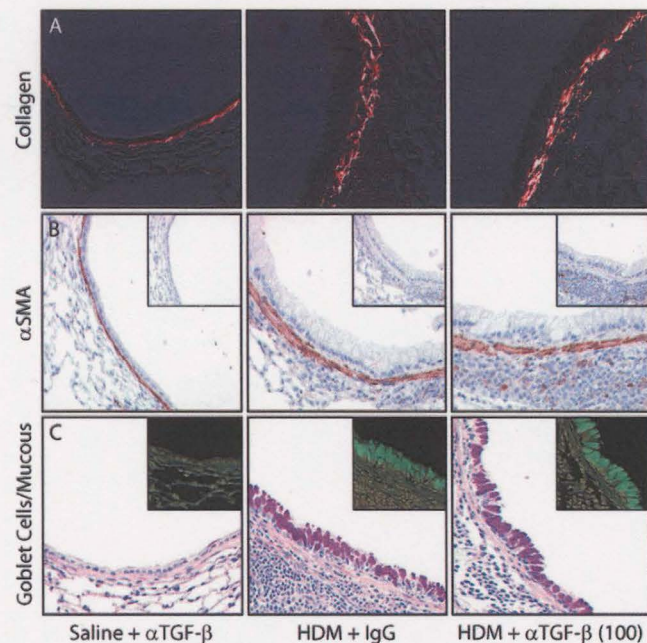


Figure 4. Impact of extended transforming growth factor (TGF)- β blockade on the development of house dust mite (HDM)-induced airway remodeling. Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) beginning at Day 4 of exposure with either a pan-neutralizing anti-transforming growth factor- β (α TGF- β ; 100 μ g/dose) antibody (Ab) or the control IgG Ab (100 μ g/dose). Panels show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition; (B) immunohistochemistry for α -smooth muscle

actin (α -SMA) indicating contractile elements in the airway wall (brown; insets show nonspecific staining in the corresponding negative control section); and (C) periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells (magenta; insets show color inverted image used for morphometric analysis). (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR, α -SMA, or PAS. All pictures were taken at $\times 20$ original magnification except insets in (C), which were at $\times 40$; $n = 6-7$ /group. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with the saline + α TGF- β -treated group.

10 μ g of α TGF- β had a slight, but nonsignificant, increase in the number of total cells and eosinophils compared with IgG-treated control animals (Figure 7A). Treatment with a higher dose of α TGF- β Ab (100 μ g/dose) significantly increased the number of total cells present in the BAL compared with HDM-exposed IgG-treated mice and, interestingly, this increase was due exclusively to a greater number of eosinophils (Figure 7A). Indeed, the proportion of eosinophils present in the BAL of mice exposed to HDM and treated with 100 μ g of α TGF- β was

nearly double that of IgG control animals. We further examined the effect of TGF- β neutralization on the inflammatory response in the context of extended Ab treatment and intermittent HDM exposure. We observed that treatment with α TGF- β over nearly the entire 5-week course of HDM administration also exacerbated the proportion of eosinophils in the BAL (Figure 7B). Notably, this extended treatment also resulted in a considerable decrease in the number of mononuclear cells (data not shown); this was not unexpected because TGF- β is

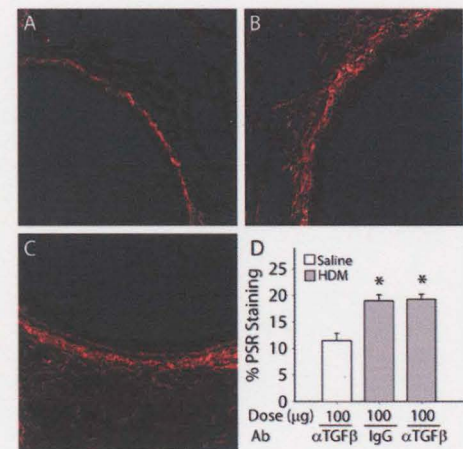
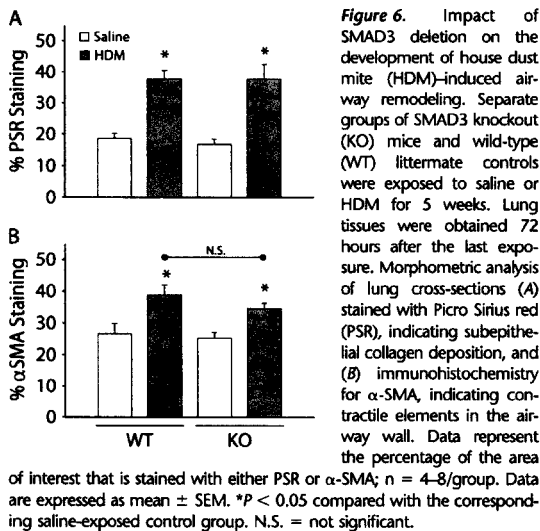


Figure 5. Impact of transforming growth factor (TGF)- β blockade on the development of house dust mite (HDM)-induced airway remodeling in a model of intermittent allergen exposure. Separate groups of mice were exposed to saline or HDM for a period of 10 consecutive days and then rested for approximately 30 days. Mice were subsequently subjected to six cycles of saline or HDM reexposure; each cycle consisted of daily exposure for 3 consecutive days followed by 12 days of rest. Mice were concurrently treated (every other day) with either anti-TGF β (α TGF β) (100 μ g/dose) or the control IgG antibody (Ab) (100 μ g/dose), over each 3-day reexposure to saline or HDM as described in METHODS. (A–C) Representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. All panels show Picro Sirius red (PSR)-stained sections visualized under polarized light indicating subepithelial collagen deposition. (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR. All images were taken at $\times 20$ original magnification; $n = 6-7$ /group. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with the saline + α TGF- β -treated group.



known to be a potent monocyte chemoattractant factor (47). Finally, consistent with the findings above, TGF-β blockade also noticeably increased the number of total cells (data not shown) and the extent of eosinophilia in the BAL of mice subjected to the intermittent HDM exposure protocol (Figure 7C), although these differences did not reach statistical significance.

Impact of TGF-β Blockade on HDM-specific Adaptive Immunity

Because TGF-β blockade led to increased airway eosinophilic inflammation, we explored whether this may have been a consequence of altered T-cell responsiveness. To evaluate T-cell function, we harvested splenocytes and measured the production of Th2-associated cytokines after stimulation with HDM *in vitro*. We observed a significant increase in the production of IL-5 by splenocytes taken from mice exposed to HDM for 5 weeks and concurrently treated with αTGF-β (100-μg dose) during the final 3 weeks compared with HDM-exposed IgG-treated control animals (Figure 8A). We also documented significantly greater IL-5 production when the duration of αTGF-β administration was increased (Figure 8B). In addition, we further noted a trend toward augmented IL-13 production in both of these experimental protocols; however, this did not reach significance (Figures 8A and 8B). No differences were observed in HDM-specific IL-4 production.

We also examined the impact of TGF-β blockade on humoral immunity. Similar to the findings above, mice treated with αTGF-β Ab during the last 3 weeks or over almost the entire course of the continuous HDM exposure protocol possessed significantly elevated serum levels of HDM-specific IgE and IgG₁ compared with the HDM-exposed IgG-treated group (Figures 8C and 8D).

Impact of TGF-β Neutralization on Lung Function

Compelled by the findings that TGF-β neutralization led to an exacerbated inflammatory response and to increased systemic immunity, we assessed the physiologic impact of TGF-β blockade on airway function. We evaluated respiratory resistance after increasing doses of methacholine in mice subjected to the continuous HDM exposure protocol and treated with αTGF-β or IgG during the last 3 weeks of HDM. Importantly, we

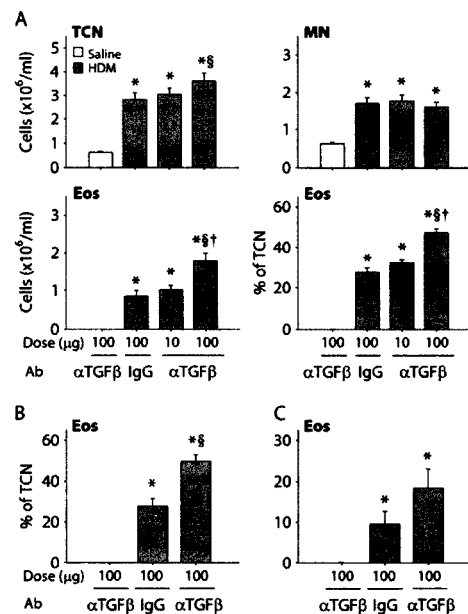


Figure 7. Impact of transforming growth factor (TGF)-β blockade on house dust mite (HDM)-induced allergic airway inflammation. (A) Differential cell analysis showing the number of total cells (TCN), mononuclear cells (MN), eosinophils (Eos), and the percentage of eosinophils in the bronchoalveolar lavage fluid of mice exposed to saline or HDM for 5 weeks and concurrently treated during the last 3 weeks of exposure with either anti-TGFβ (αTGF-β) (10 or 100 μg/dose) antibody (Ab) or the control IgG (100 μg/dose). (B) Percentage of eosinophils in the BAL fluid of mice exposed to saline or HDM for 5 weeks and concurrently treated beginning at Day 4 of exposure with either αTGF-β (100 μg/dose) or the control IgG (100 μg/dose). (C) Percentage of eosinophils in the BAL fluid of mice subjected to the intermittent allergen exposure and treated with either αTGF-β (100 μg/dose) or the control IgG (100 μg/dose) according to the protocol shown in Figure 1C. n = 14–21/group in (A) and n = 5–7/group in (B) and (C). Data are expressed as mean ± SEM. P < 0.05 compared with *saline + αTGF-β-, §HDM + IgG-, and †HDM + αTGF-β (10 μg/dose)-treated groups, respectively.

observed that HDM-exposed αTGF-β-treated mice exhibited a trend toward increased maximum resistance (Figures 9A and 9B) and significantly greater airway reactivity versus HDM-exposed IgG-treated mice (Figures 9A and 9C).

DISCUSSION

In light of the mounting evidence implicating TGF-β in the pathogenesis of asthma, we investigated the impact of interfering with TGF-β activity on the regulation of chronic allergic airway inflammation and remodeling induced by exposure to HDM. We observed that treatment with a neutralizing αTGF-β Ab had no effect on the development of airway remodeling, even if the dose of Ab was increased 10-fold and treatment was extended over nearly the entire course of HDM exposure. These observations suggested to us that the remodeling response, at least when triggered by continuous HDM exposure, could occur independently of TGF-β activity. Similarly, treatment with

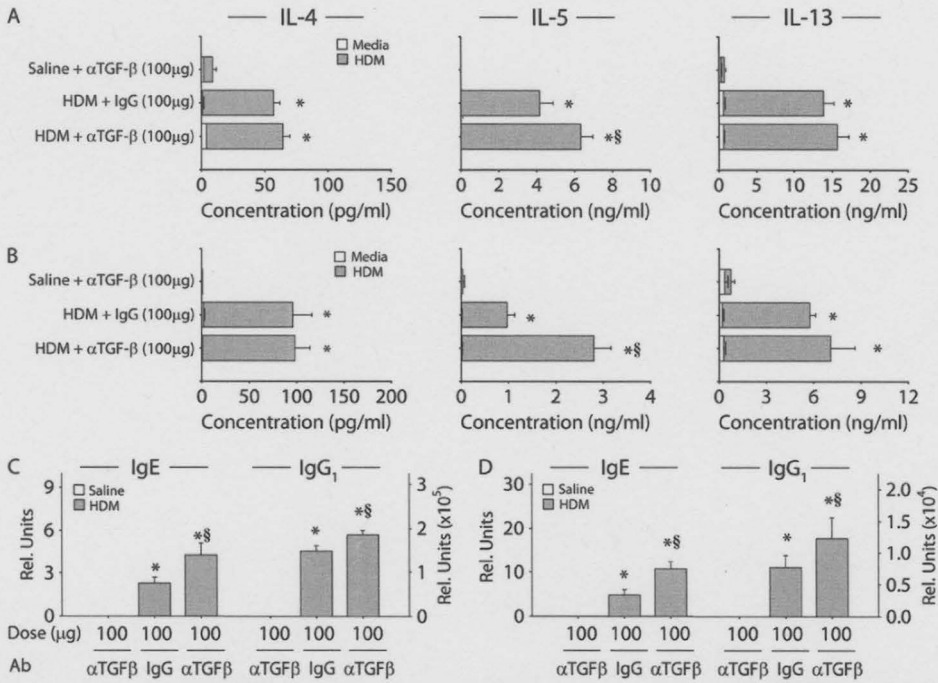


Figure 8. Impact of transforming growth factor (TGF)-β blockade on house dust mite (HDM)-specific Th2-associated immune responses. HDM-specific Th2-associated splenocyte cytokine production (A and B) and serum immunoglobulin levels (C and D) from mice exposed to saline or HDM for 5 weeks and concurrently treated during the last 3 weeks of exposure (A and C) or beginning at Day 4 of exposure (B and D) with either anti-TGFβ (αTGF-β) (100 μg/dose) antibody (Ab) or the control IgG Ab (100 μg/dose). Splenocytes from individual mice were cultured in medium alone (open bars) or stimulated with HDM (shaded bars) *in vitro*. n = 5–7/group. Data are expressed as mean ± SEM. P < 0.05 compared with *saline + αTGF-β- and §HDM + IgG- treated groups, respectively.

αTGF-β in the context of an intermittent HDM exposure protocol, in which the ensuing inflammatory response is recurrent and therefore considerably lower, also had no effect. This argued against the possibility that TGF-β was dispensable in the continuous HDM exposure protocol simply because that protocol elicits a robust inflammatory response that is sustained over several weeks. Moreover, these findings suggest that other factors, such as the nature of the eliciting agent and/or the type of the inflammatory response, may ultimately influence whether TGF-β is required in a given reparative/fibrotic response. Indeed, that the type of inflammation impacts whether a reparative/fibrotic response will develop has been previously demonstrated (14, 48, 49).

To further confirm the observations made with the blocking Ab, we used a second approach, the use of SMAD3 KO mice, to evaluate the role of TGF-β in HDM-induced remodeling. The SMAD signaling pathway is not the only pathway by which the fibrogenic effects of TGF-β may be mediated (50); however, SMAD3 has been clearly shown to play a fundamental role in the signal transduction pathways associated with TGF-β-mediated wound healing and fibrosis (46). In agreement with the findings above, we observed that HDM-exposed SMAD3 KO mice developed airway remodeling to the same extent as WT littermate controls. Thus, when taken together, the findings we report here demonstrate that HDM-induced allergic airway remodeling can develop independently of TGF-β.

Although our data may initially appear to be at variance with the prevailing notion that TGF-β mediates, in all circumstances,

tissue repair, careful consideration of the differences between the various systems used may in fact account for these seemingly divergent findings. In the case of fibrogenesis, a common feature of models of inflammation and fibrosis is that there is a single eliciting event (e.g., bleomycin, radiation, administration of TGF-β). This generates an acute and self-limited inflammatory process that is associated with the development of fibrosis. These approaches remarkably contrast with our model in that the eliciting agent (HDM) is delivered continuously for a considerable period of time. In addition, the type of inflammation (Th2 polarized) is yet another central difference between our model and most models of fibrosis.

The findings by McMillan and colleagues warrant particular consideration as they investigated the impact of TGF-β neutralization, using the same Ab as the one used here, in a model of chronic allergic airway disease (18). They showed that TGF-β neutralization prevented the progression of airway remodeling after repeated OVA challenge. However, there are several important considerations that must be taken into account. First, one should note that, whereas this treatment indeed prevented airway remodeling at an early time point (Day 35) of their protocol, the effects of TGF-β neutralization on the extent of remodeling at a later time point (Day 55) were substantially reduced, suggesting, as the authors noted, that additional factors are likely contributing to the development of airway remodeling in their model. Second, as is well known in models using OVA, repeated OVA challenge leads to a diminution of the inflammatory response, as certainly occurred in McMillan and

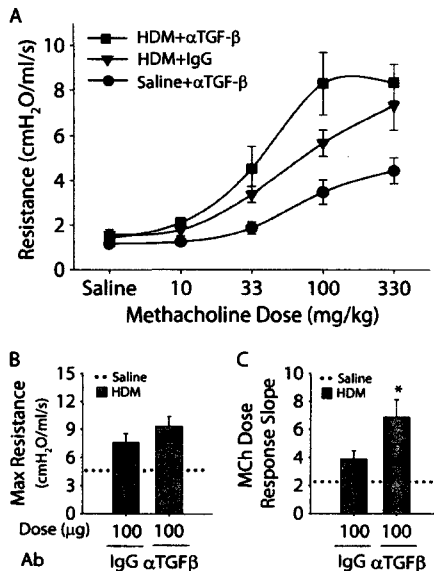


Figure 9. Impact of transforming growth factor (TGF)- β blockade on house dust mite (HDM)-induced airway responsiveness to methacholine (MCh). (A) Total respiratory resistance was measured at increasing doses of MCh in separate groups of mice exposed to saline or HDM for 5 weeks and concurrently treated during the last 3 weeks of exposure with either anti-TGF β (α TGF- β) (100 μ g/dose) antibody (Ab) or the control IgG Ab (100 μ g/dose). (B) Maximum respiratory resistance values and (C) airway reactivity as measured by maximum resistance and MCh dose-response slope, respectively, for mice exposed to saline (dotted line) or HDM (shaded bars) and concurrently treated during the last 3 weeks of exposure with either α TGF- β (100 μ g/dose) or the control IgG Ab (100 μ g/dose). $n = 6-7$ /group. Data are expressed as mean \pm SEM. $P < 0.05$ compared with *HDM + IgG-treated group.

colleagues' protocol. This is in sharp contrast with the model that we used in which we maintain robust inflammation (30–35% eosinophils in BAL) for several weeks. Third, it may be particularly important that the antigen used in this model was OVA, a prototypic innocuous antigen with fundamental biochemical differences to HDM. Indeed, whereas OVA preparations are essentially pure, HDM is, in stark contrast, a complex material consisting of numerous protein and nonprotein components, with considerable proteolytic activity. This added complexity may be of considerable relevance because the biochemical and immunogenic profile of HDM will likely draw on a distinct network of molecular responses. Therefore, we argue that, whereas OVA can, under certain conditions (e.g., intraperitoneal sensitization with intermittent exposure) lead to remodeling, achieving the same outcome using HDM and a route of mucosal sensitization does not imply the same underlying biochemical or immunologic process; nor can this knowledge be universally applied to the responses elicited by other aeroallergen families. Thus, from this perspective, we do not view our findings to be in contrast with those described above but instead to be a reflection of the differences in the contexts in which the role of TGF- β is being explored.

Particular attention should also be drawn to the study by Kaviratne and coworkers (51). There they comprehensively investigated the requirement for TGF- β in the generation of

liver fibrosis induced by *Schistosoma mansoni* infection. Most interesting, this fibrotic response develops as a consequence of a Th2-polarized inflammatory response directed against schistosome eggs, which get trapped in the liver. Using an exhaustive array of methodologies, the authors conclusively demonstrated that the fibrotic response induced by schistosome infection was TGF- β independent. Notably, IL-13, a prototypical Th2-associated cytokine, was shown to be an indispensable mediator of hepatic fibrosis in this model (52). Interestingly, constitutive IL-13 overexpression in the lung has been shown to trigger a Th2-like response involving airway eosinophilia and hyperactivity that was furthermore associated with the development of subepithelial airway fibrosis (53). However, in contrast to the observations made in the schistosome model, the fibrotic response that develops as a consequence of IL-13 overexpression was found to be mediated by TGF- β , because administration of a soluble TGF- β receptor-Fc molecule ameliorated IL-13-induced fibrosis (54). The difference in the requirement for TGF- β between these two models may reflect the fact that IL-13 overexpression does not fully recapitulate a Th2-polarized immune response. Indeed, many of the other hallmark Th2-associated cell types and molecules (B cells, Th2 cells, IL-4, IL-5, IL-9, and IgE) were absent in the transgenic IL-13 model. These additional components are likely to critically influence the lung microenvironment and therefore may alter the requirement for TGF- β in the generation of a fibrotic response that develops in the context of an antigen-dependent Th2 immune response. Taken together, these studies support the concept that some Th2-associated airway reparative responses occur independently of TGF- β , and moreover, that the molecular signatures underlying the development of a "fibrotic event" may be critically influenced by the type of inflammatory response, and therefore by the nature of the eliciting agent (e.g., bleomycin, radiation, schistosoma, OVA, HDM). Thus, it follows that there may be several distinct pathways leading to fibrosis—that is, a TGF- β -dependent pathway and an IL-13-dependent TGF- β -independent pathway, possibly among others. In addition, there exists a panoply of other molecules that display powerful fibrogenic activity. These include platelet-derived growth factor, vascular endothelial growth factor, connective tissue growth factor, and oncostatin M, among several others (55, 56). The precise contribution of these molecules, either alone or in concert, in mediating tissue reparative responses associated with Th2-polarized airway inflammation remains to be fully dissected.

We also investigated the impact of interfering with TGF- β activity on the allergic inflammatory response. We observed that TGF- β neutralization, when initiated at a time when the inflammatory response was already ongoing (2 wk of HDM exposure), led to a significant increase in inflammation that was characterized exclusively by an increase in eosinophils; similar effects were also evident when TGF- β neutralization was initiated early on during continuous HDM exposure (Day 4) and in the intermittent exposure protocol, although the increase did not reach statistical significance in the latter. These increases could be the result of augmented IL-5 production, although it is also plausible that α TGF- β treatment interfered with TGF- β -induced eosinophil apoptosis, which has been previously observed *in vitro* (30). We surmise that the increases in eosinophilia likely underlie the enhanced airway reactivity we observed. Similar to our findings, TGF- β neutralization was recently reported to result in increased airway hyperresponsiveness in an OVA-based model (20). Although this occurred in the absence of any noticeable effects on inflammation, this study (in agreement with others) showed that TGF- β neutralization led to increased production of Th2 cytokines. Thus, it is also plausible that the increase in airway hyperresponsiveness may be a result of increased levels of IL-5

and/or IL-13, among others, via some direct effect(s) on, for example, airway smooth muscle cells. Ultimately, as elegantly discussed by Alcorn and colleagues (20), a number of intertwined mechanisms could account for this observation. Importantly, TGF- β blockade, at least in the context of continuous HDM exposure, carried significant functional consequences.

Several related studies using acute conventional OVA models (33–35) have also documented increases in airway inflammation attributable in large part to the following: enhanced eosinophilia; elevated levels of Th2-associated cytokines, including IL-4, IL-5, and IL-13; and increased serum IgE and IgG₁ in mice in which TGF- β levels or activity had been impaired. Although it was not possible to clearly identify the effects on established inflammation alone given the genetically based methods these authors used, their data implicate TGF- β as an important regulator of allergic airway inflammation. Interestingly, in both the McMillan and Alcorn studies, TGF- β neutralization in their models had no effect on established inflammation. We suspect that this observation may be related to the dose of Ab used and/or the system used and not necessarily a reflection of the role of TGF- β in the regulation of chronic allergic inflammation. Using a similar dose of Ab used in McMillan and colleagues' protocol, we observed a clear trend toward enhanced eosinophilia. Administration of a higher dose of Ab conclusively demonstrated, at least in our system, that TGF- β is a critical regulator of HDM-induced chronic allergic airway inflammation.

In our view, the findings we present here do not question the importance of TGF- β to the tissue repair response. Rather, they compel us to consider the notion that the role of a given molecule in a process is contextual. In the context of robust airway inflammation elicited by persistent exposure to a common aeroallergen (i.e., HDM), the research we present here demonstrates that TGF- β does not play a critical role in airway remodeling, which, we understand, may be divergent from the prevailing dogma. In addition, our data show that interference with TGF- β leads to worsened airway inflammation and function. We surmise that these findings are, at least teleologically, in accord with the principal goal of the immune response: survival of the host. That is, that the key role of TGF- β under these conditions may be to protect the host from devastating inflammation and tissue damage. From this perspective, an important concern arises with respect to the proposition of therapeutic interference with TGF- β as a means of limiting the progression of airway remodeling, not only that it may not work but also that the treatment may lead to a loss of control of the inflammatory response and, ultimately, the disease.

Conflict of Interest Statement: R.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.G.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.R.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.D.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.P.S. is an employee of Genzyme Corporation and owns shares of Genzyme Corporation common stock. R.C.G. is an employee of Genzyme Corporation and owns shares of Genzyme Corporation common stock. S.L. is a salaried employee of Genzyme Corporation and has shares of Genzyme Corporation common stock. J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Online Data Supplement

TGF- β Regulates House Dust Mite-Induced Allergic Airway Inflammation But Not Airway Remodeling

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Materials and Methods:

Collection and Measurement of Specimens

Bronchoalveolar lavage (BAL) fluid, lungs, blood, and spleen were collected at the time of sacrifice. BAL was performed as previously described (E1). Briefly, lungs were dissected and the trachea was cannulated with a polyethylene tube (outer/inner diameter = 0.965/0.58mm; Becton Dickinson, Sparks, MD, USA). Lungs were lavaged twice with PBS (0.25ml followed by 0.2ml) and approximately 0.25-0.3ml of the instilled fluid was consistently retrieved. Total cell counts were then determined using a hemocytometer. Each BAL sample was then centrifuged and the supernatants collected and stored at -20°C. Cell pellets were subsequently resuspended in PBS and smears were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, PA, USA) at 300rpm for 2min. Protocol Hema 3 stain set (Fisher Scientific, Toronto, ON, Canada) was used to stain all smears. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Where applicable, following BAL, the right lung was dissected and placed in PBS at 4°C for tissue homogenate preparation; the left lung was inflated with 10% formalin at a constant pressure of 20cm H₂O, and then fixed in 10% formalin for 48-72 hours. Peripheral blood was collected by retroorbital bleeding and serum was obtained by centrifugation of whole blood after 30min incubation at 37°C and stored at -20°C. Harvested spleens were placed in sterile tubes containing sterile HBSS.

Preparation of Lung Tissue Homogenate and Hydroxyproline Measurement

The right lung was homogenized in 1.5 ml of TNE buffer (25 mM Tris, 125 mM NaCl and 2.5 mM EDTA) supplemented with 40 µl of sodium orthovanadate and 1 COMPLETE protease

inhibitor tablet (Roche, Laval, QC, Canada) per 20 ml of buffer. Following homogenization, 150 μ l of 10% TritonX-100 was added and samples were rocked at 4°C for 1 hour. Supernatant was collected following a 15 minute spin at 12,000 rpm at 4°C and stored at -70°C. For the hydroxyproline assay, 500 μ l of supernatant was freeze dried and the dry tissue weight was measured. Dry tissue was then resuspended in 2 ml of PBS, transferred to a glass pyrex tube, protein was precipitated using 200 μ l of 50% TCA at -20°C for 30 minutes and tubes were spun for 30 mins at 1800 rpm. Supernatant was then discarded and protein was hydrolyzed in 2 ml of 6N HCl for 16 hours at 105-110°C. This solution was then neutralized by titration using 10N/1N NaOH and 100 μ l of methyl red indicator. The total volume of NaOH added was recorded and taken into account in the final calculation. A 400 μ l aliquot of the titrated solution was then brought up to a total volume of 2 ml with ddH₂O and hydroxyproline content was determined by colourimetric assay as described in (E2). Results were calculated as μ g of hydroxyproline per mg of dry lung weight using hydroxyproline standards (Sigma).

Histology and Immunohistochemistry

Following formalin fixation, tissues were embedded in paraffin and 3- μ m-thick cross-sections of the left lung were cut and stained with hematoxylin and eosin (H&E), Picro Sirius red (PSR), and periodic acid-Schiff (PAS). To detect the presence of airway smooth muscle, immunohistochemistry (IHC) was performed. Paraffin sections of lungs were deparaffinized, followed by 100% ethanol and then placed in freshly prepared methanol H₂O₂ solution to block endogenous peroxidase. Following hydration, heat-induced epitope retrieval was performed using 10 mM citrate buffer (pH 6.0) in a steamer for 45 minutes. Protein block was performed by incubating sections in 1% v/v normal swine serum (NSS) for 15 minutes. Sections were then incubated with a purified mouse monoclonal anti- α -smooth muscle actin

primary antibody (Clone 1A4, Dako, Denmark; 1:150 in UltraAb Diluent, Labvision, Fremont, CA, USA) for 1 hour; negative diluent control sections were run in parallel with the primary incubation. All sections were then incubated with biotinylated rabbit anti-mouse secondary antibody (1:300 in 1% v/v NSS; Dako) for 1 hour at room temperature (RT), followed by streptavidin-peroxidase conjugate (1:600 in 1% v/v NSS; Dako) for 45 minutes at RT. Sections were placed in acetate buffer (pH 5.0) for 5 minutes and then incubated in freshly prepared AEC chromogen substrate solution for 15 minutes. Tissues were counterstained in Mayer's hematoxylin for 1 minute, washed, and mounted in glycerin gelatin. Appropriate washes were performed between steps using 0.05 M Tris buffered saline. Some non-specific binding of the primary Ab is commonly observed in tissue sections as both the Ab and the tissue sections are from the same species (i.e. mouse). We have generally observed that non-specific staining does not interfere with quantitative analysis. Regardless, negative controls are generated for each individual tissue section and any non-specific staining in the region of interest is taken into account during morphometric analysis.

Morphometric Analysis

Images for morphometric analysis were captured with OpenLab software (v3.0.3; Improvision, Guelph, ON, Canada) via a Leica camera and microscope. Image analysis was performed using a custom computerized analysis system (Northern Eclipse software v5; Empix Imaging, Mississauga, ON, Canada). Analysis of sections stained for α SMA, PSR and PAS were performed as previously described (E3). Distances of 20 and 40 μ m (starting from below the airway epithelium and proceeding away from the lumen) were used for α SMA and PSR images respectively, while a distance of 30 μ m (starting from below the airway epithelium and proceeding towards the lumen) was utilized for PAS stained images.

Splenocyte Culture

Splenocytes were isolated by triturating whole spleens through a 40µm cell strainer (Becton Dickinson) using the plunger from a 5ml syringe into HBSS. Following centrifugation at 1200rpm for 10min at 4°C, red blood cells were lysed using ACK lysis buffer (0.5 M NH₄Cl, 10 mM KHCO₃, and 0.1 nM Na₂EDTA, pH 7.2-7.4). Splenocytes were then washed once with HBSS and then again with RPMI supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen, Grand Island, NY, Canada), and 0.1% β-mercaptoethanol (Invitrogen). Splenocytes were then resuspended in complete RPMI at a concentration of 8x10⁶ cells/ml and cultured in medium alone, or with medium supplemented with HDM (31.25µg/ml) in a flat-bottom, 96-well plate (Becton Dickinson), in triplicate; 100µl of cell suspension + 100µl of medium+HDM per well. After 5 days of culture, supernatants were harvested and triplicates were pooled for cytokine measurements.

Cytokine and Immunoglobulin Measurements by ELISA

DuoSet ELISA kits for mouse interleukin (IL)-4, -5, -13 and active TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA) and used according to the manufacturer's instructions. Each of these assays has a threshold of detection between 1.5-5pg/ml. For detection of HDM-specific IgE, Maxi-Sorp plates (NUNC Brand Products, Denmark) were coated with 100µl/well of a purified rat IgG₁κ mAb against the ε heavy chain of mouse Ig (LO-ME-3; University of Louvain, Belgium) at a concentration of 5 µg/mL in borate buffer (pH 8.3-8.5) overnight at 4°C. Coating solution was then aspirated (without washing) and wells were subsequently blocked with 100µl of 3% normal mouse serum (NMS; Sigma) for 2 hours at RT and washed. 50 µL of sample (diluted 2x in 3% NMS/PBS) was then added to each well and incubated overnight at 4°C. Following a wash, 50µL of biotinylated HDM (diluted in 3%

NMS/PBS) was then added to each well, incubated at RT for 2 hours and washed again. For detection of HDM-specific IgG₁, Maxi-Sorp plates were coated with 5 µg of HDM in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Subsequently, coated wells were blocked with 1% w/v BSA in PBS for 2 hours at RT. After washing, serum samples (diluted 1:20, 200, 2000 and 20,000 in 0.3% BSA/PBS – 50µl/well) were incubated overnight at 4°C, washed and then incubated with 0.25µg/ml biotin-labeled IgG₁ (Southern Biotechnology Associates) overnight at 4°C. For both HDM-specific IgE and IgG₁, plates were then incubated with alkaline-phosphatase streptavidin (Zymed, South San Francisco, California, USA), 50µl/well, at a concentration of 1:1000 for 1 hour at RT and washed. The color reaction was developed with *p*-nitrophenyl phosphate (Sigma) in 10% v/v diethanolamine buffer (Sigma), 50µl/well, and stopped with 25µl/well of 2N NaOH. ODs were read at 405 nm. Washes were performed with 300ul of 0.05% v/v Tween 20 in PBS and the 'blank' OD value was based on the average of 20 control wells that were loaded with diluent instead of sample. For HDM-specific IgE and IgG₁, units correspond to the maximal dilution that resulted in an OD (for most positive samples) which exceeded the average OD value of 20 zero standard replicates plus two standard deviations. Formula used to calculate Relative Units: Titre = $1/[(\text{dilution of OD reading})/(\text{OD reading}) \times (0.05)]$.

For detection of αTGF-β murine mAb in the BAL, serum and LG homogenate samples, 96-well plates were coated overnight at 4°C with purified TGF-β₂ (1 µg/mL in 0.1M sodium carbonate buffer pH 9.2). Subsequently, the plate was washed and wells were blocked using a 1% BSA/dH₂O solution for 1 hour at 37°C. After washing, samples were diluted in PBS supplemented with 0.05% Tween 20, 0.1% BSA and 0.05% Triton-X, added to the wells and incubated for 2 hours at 37°C. Samples were then washed again, and bound αTGF-β Ab was

detected using a goat anti-mouse IgG-HRP (Fc specific) Ab (Sigma-Aldrich, St. Louis, MO, USA; 1 hr incubation at 37°C). TMB microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was then added to each well and ODs were read at 450nm. Concentration of α TGF- β in the sample was determined on the basis of comparison to a standard curve generated using several dilutions of a known concentration of α TGF- β Ab. All steps (except washes) were carried out in 100 μ L volumes.

Bioassay for TGF- β

Bioactive TGF- β was measured in the BAL fluid using the plasminogen activator inhibitor-1 (PAI-1) luciferase-based TGF- β bioassay, as previously described in (E4). Briefly, mink lung epithelial cells (MLECs, kindly provided by D. Rifkin, New York, NY), carrying a stable transfection of an 800-bp fragment of the 5' end of the human PAI-1 gene fused to the firefly luciferase reporter gene, were cultured in 100 mm dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 250 μ g/ml geneticin (culture medium) until confluent. Cells were then harvested, washed, and 100 μ l of a 1.6×10^5 cells/ml solution was plated in a flat-bottom 96-well plate. Cells were allowed to adhere for 4-6 hours. Culture medium was then removed and 100 μ l of standards (using rhTGF- β ; R&D Systems) and BAL samples were plated in triplicate (where possible) and incubated at 37°C for 14-16 hours. BAL samples were diluted 3x using DMEM supplemented with 0.2% pyrogen-poor BSA (Sigma) and 1% L-glutamine (assay medium). Standards were prepared in diluted assay medium (67% assay medium:33% PBS). Following incubation, cells were washed with PBS and a lysate prepared by adding 100 μ l of luciferase cell lysis buffer (BD Pharmingen) with shaking for at least 30 minutes. Lysates were then assayed for luciferase activity using a luminometer (Tropix).

Airway Responsiveness Measurements

Airway responsiveness was measured on the basis of the response of total respiratory system resistance to increasing i.v. (internal jugular vein) doses of methacholine (MCh) as previously described (E5, E6). Evaluation of airway responsiveness was based on the peak respiratory resistance measured in the 30s after the saline and MCh challenges. Briefly, mice were anesthetized with tribromoethanol (287 mg/kg i.p.), prepared according to a standard protocol (E7). The trachea was exposed and cannulated, and a constant inspiratory flow was delivered by mechanical ventilation (RV5; Voltek Enterprises Inc., Toronto, Ontario, Canada). Heart rate and oxygen saturation were monitored via infrared pulse oximetry (Biox 3700; Ohmeda, Boulder, Colorado, USA), using a standard ear probe placed over the proximal portion of the mouse's hind limb. Paralysis was achieved using pancuronium (0.03 mg/kg intravenously) to prevent respiratory effort during measurement. Respiratory resistance was measured after consecutive intravenous injections of saline, followed by 10, 33, 100 and 330 $\mu\text{g}/\text{kg}$ of MCh (ACIC [Can], Brantford, Ontario, Canada), each delivered as a 0.2 ml bolus. During each MCh dosing, the mouth-pressure signal from the ventilator was converted to a digital signal (Dash 16; Metrabyte, Staughton, Massachusetts, USA) and recorded on a PC. Respiratory resistance was calculated as described previously (E6). Airway responsiveness was quantified by the slope of the linear regression between peak respiratory resistance and the \log_{10} of the MCh dose using the data from the 10, 33 and 100 $\mu\text{g}/\text{kg}$ doses only.

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Figure Legends:

Figure E1. Levels of bioactive TGF- β protein in the BAL. Graph shows the concentration of bioactive TGF- β in the BAL fluid of mice exposed to saline or HDM for 5 weeks and concurrently treated (qod) during the last 3 weeks of exposure with either α TGF- β (100 μ g/dose) or the control IgG (100 μ g/dose). Equivalent volumes of BAL fluid from individual mouse samples were pooled within groups ($n = 4-5$ /group) and run in triplicates (where possible). Bioactive TGF- β was measured using the plasminogen activator inhibitor-1 luciferase (PAI/L)-based TGF- β bioassay. Data are expressed as the mean \pm SD of triplicates (where applicable).

Figure E2. Total lung hydroxyproline content. Mice were exposed to saline or HDM for 5 weeks and concurrently treated (qod) beginning at day 4 of exposure with either α TGF- β (100 μ g/dose) or the control IgG Ab (100 μ g/dose). Lung homogenates were prepared and hydroxyproline content (indicative of lung collagen content) was measured as described in the METHODS. The results were calculated as μ g hydroxyproline per mg dry lung tissue weight. $n = 5-7$ /group. Data are expressed as mean \pm SEM. $p < 0.05$ compared to *saline + α TGF- β treated group.

FIGURE E1

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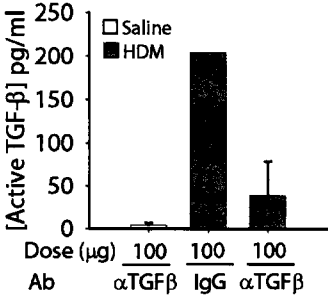
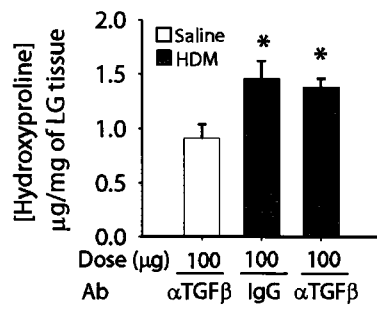


FIGURE E2

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— Chapter 4 —

Eosinophils are Dispensable for House Dust Mite-Induced Allergic Airway Remodeling and Immunity

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See *Preface* for details regarding authorship.

Summary and Central Message: This article extends the work presented in the previous chapter. Here, we investigate whether eosinophils contribute to the development of HDM-induced remodeling and immune-inflammatory responses. To eliminate eosinophils we employed two distinct strategies, first, the use of an anti-CCR3 eosinophil-depleting Ab and second, two separate strains (Δ dblGATA and *PHIL*) of eosinophil-deficient mice. We show that GATA and anti-CCR3 treated WT mice subjected to chronic HDM exposure developed airway remodeling to the same degree as that observed in WT HDM-exposed mice. A similar outcome was also documented in *PHIL* eosinophil-deficient mice exposed to HDM, although, in this case a slight decrease in some remodeling-associated parameters were noted. In addition, we consistently found that the absence of eosinophils had no impact on the induction of HDM-specific Th2-polarized adaptive immunity and only modestly affected airway inflammation. When taken together, these findings suggest that eosinophils augment allergic airway inflammation but, are largely dispensable for the generation of HDM-induced allergic remodeling and adaptive immunity. Moreover, they further underscore the notion that the role of a given factor in allergic disease may be context specific and not broadly applicable to the responses elicited by all aeroallergens.

Eosinophils are Dispensable for House Dust Mite-Induced Allergic Airway Remodeling and Immunity¹

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“At a Glance Commentary”

The current dogma, based largely on experimental evidence from studies employing conventional ovalbumin-based models of allergic airway disease, is that eosinophils play a prominent role in the development of airway remodeling, a characteristic feature of chronic allergic asthma. This research contributes to our understanding of the mechanisms regulating Th2-polarized inflammation, immunity and remodeling in chronic allergic asthma, and suggests that therapeutic strategies to prevent remodeling through eosinophil depletion may have negligible effects.

Running Title: Eosinophils in HDM-induced Allergic Remodeling

Key Words: airway remodeling, house dust mite, allergic asthma, eosinophils, mouse

ABSTRACT

Rationale: Current thinking accredits eosinophils with preeminent contributions to allergic airway responses including a major role in the development of airway remodeling, a process thought to significantly contribute to airway dysfunction. However, direct evidence in support of this notion is limited and often controversial. *Objectives:* Here, we elucidated the requirement for eosinophils in the generation of allergic sensitization, airway inflammation and remodeling in a model involving chronic respiratory exposure to house dust mite (HDM). *Methods:* We employed three methods to selectively eliminate eosinophils, a depleting antibody (anti-CCR3) and two strains of eosinophil-deficient mice (Δ dblGATA and the transgenic line *PHIL*). *Measurements and Main Results:* We report that anti-CCR3 treatment markedly reduced pulmonary eosinophilia (>80%) over the course of HDM exposure, but had no effect on the remaining inflammatory response, extent of lung Th2 cells or development of remodeling-associated changes including subepithelial collagen deposition and smooth muscle thickening. In addition, we observed that, despite the complete absence of eosinophils, HDM-exposed GATA mice mounted robust airway/lung inflammation and hyperresponsiveness, and a remodeling response equivalent to that observed in wild-type mice. Moreover, these mice had similar serum HDM-specific IgE levels and Th2-associated splenocyte cytokine production as HDM-exposed wild-type controls. Similar observations were made in *PHIL* eosinophil-deficient mice subjected to chronic HDM exposure, although slight decreases in airway mononuclear cells, but not lung Th2 cells, and remodeling were noted. *Conclusions:* Collectively, these data demonstrate that, at variance with the prevailing paradigm,

eosinophils play negligible roles in the generation of HDM-induced allergic immunity and airway remodeling.

INTRODUCTION

Allergic asthma is a chronic immune-inflammatory driven disease that involves the development of various alterations to the structure of the airway walls. These alterations, collectively termed *airway remodeling*, include: i) goblet cell hyperplasia and increased epithelial cell turnover and mucous production; ii) thickening of the extracellular matrix (ECM) below the epithelium due to increased deposition of matrix proteins such as collagen, fibronectin, and laminin, among others; iii) increased smooth muscle mass on account of smooth muscle cell hypertrophy and/or hyperplasia; and iv) an increase in the small blood vessel area (1, 2). Although not exempt of controversy, the evidence regarding the clinical and functional impact of remodeling indicates that at least some elements of it play a significant contribution (2, 3).

Various cell types, both structural and immune, have been implicated in airway remodeling. In particular, eosinophils are thought to be principally involved as they are able to produce a multitude of cytokines, growth factors and toxic mediators that can influence the function, proliferation and differentiation of resident structural lung cells and, thus, could drive airway remodeling. Experimental studies employing interleukin (IL)-5 and eotaxin overexpressing transgenic mice have shown that eosinophils *can* elicit many of the hallmark pathologies of allergic airway disease including airway remodeling

(4). However, whether eosinophils actually *do* contribute to the remodeled airway remains debatable. Additional investigations in mouse models examining the requirement for eosinophils in the development of airway remodeling, indirectly, by interfering with IL-5 function have yielded disparate results (5-10). Interestingly, significant reductions in subepithelial collagen deposition and smooth muscle cell proliferation were observed in sensitized and chronically challenged Δ dblGATA eosinophil-deficient mice compared to wild-type (WT) controls (11), suggesting that eosinophils may play a central role in remodeling. A common feature of these studies is that they all employed ovalbumin (OVA)-based models. This, we surmise, is of particular importance as a number of key differences, mechanistic and otherwise, are emerging between OVA-based models and models employing a common environmental allergen (CEA). For example, while transforming growth factor (TGF)- β has been shown to be an integral mediator of remodeling in the OVA systems (12-14), TGF- β was not critically required for the development of house dust mite (HDM)-induced remodeling (15). To date, the role of eosinophils in remodeling has not been examined in an experimental system using a CEA. Thus, in this report we comprehensively investigated the role of eosinophils in HDM-induced allergic airway remodeling and adaptive immunity. Our data conclusively show that eosinophils play a negligible role in these processes.

Some of the results of these studies have been previously reported in the form of an abstract (16).

METHODS

Animals

Female WT BALB/c mice (8 weeks old) were purchased from Charles River Laboratories (Ottawa, ON, Canada). Δ dblGATA eosinophil-deficient mice (BALB/c background) were bred in-house. *PHIL* eosinophil-deficient male mice (8th generation BALB/c background; courtesy of Dr. J.J. Lee) were mated with WT BALB/c females purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Female *PHIL* eosinophil-deficient mice (8 weeks old) and their WT littermate controls were employed in the experiments described herein. The genotypes of both WT littermate and *PHIL* eosinophil-deficient mice were confirmed by PCR analysis on tail DNA as previously described (17). All mice were housed under specific pathogen-free conditions and maintained on a 12-hour light-dark cycle, with food and water ad libitum. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada).

Sensitization Protocol

Allergen administration - House dust mite extract (HDM; Greer Laboratories, Lenoir, NC, USA) was resuspended in sterile saline (2.5mg of protein/ml) and 10 μ l was administered intranasally (i.n.) to isoflurane-anaesthetized mice. Separate groups of mice were exposed to HDM or saline for 5 consecutive days followed by 2 days of rest for a total of 4 or 5 weeks depending on the experiment and were sacrificed 72 hours after the last HDM/saline exposure.

Antibody administration – Where applicable, a rat anti-mouse-CCR3 monoclonal antibody (Ab; anti-CCR3 hybridoma courtesy of Yonghong Wan, McMaster University, and DYNAX research institute) or a control Ab (total rat IgG; Sigma, Oakville, ON, Canada) was repeatedly administered to separate groups of anesthetized mice, intraperitoneally (i.p.), at a dose of 0.5mg/injection in 0.5ml of sterile phosphate-buffered saline (PBS) immediately prior to HDM administration. Mice were injected on days 3, 6, 9, 13, 16, 19, 22, and 25 of the HDM administration protocol.

Collection and Measurement of Specimens

Bronchoalveolar lavage (BAL), lungs, blood and spleen were collected approximately 72 hours after the last HDM challenge. See the online supplement for additional details on the methods used to perform BAL. Protocol Hema 3 stain set (Fisher Scientific, Toronto, ON, Canada) was used to stain all smears. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as eosinophils, mononuclear cells or neutrophils. Where applicable, following BAL, the lungs were perfused through the right ventricle with 10ml of warm Hank's balanced salt solution (HBSS) and then the right lung was dissected and placed into HBSS at 4°C for lung cell isolation. The left lung was inflated with 10% formalin at a constant pressure of 20cm H₂O and then fixed in 10% formalin for 48-72 hours for histochemical and morphometric analysis. Peripheral blood (PB) was collected by retroorbital bleeding. Serum was obtained by centrifugation of whole blood after 30min incubation at 37°C and stored at -20°C. For PB leukocyte (PBL) isolation, up to 1ml of blood was collected in a

tube containing 50µl of 100mM ethylenediaminetetraacetic acid (EDTA) in PBS (pH 7.2-7.4). Harvested spleens were placed in sterile tubes containing sterile HBSS.

Histology and Immunohistochemistry

Following formalin fixation the left lung was cut, cross-sectionally, immediately below the bronchial entry point and again approximately 5mm below the first cut yielding 3 separate pieces. Tissues were embedded in paraffin and 2 separate 3-µm-thick cross-sections (>100µm apart) were cut and stained with either Picro Sirius red (PSR) or Periodic acid-Schiff (PAS). Immunohistochemistry for α -smooth muscle actin (α -SMA) was also performed as previously described in detail (15).

Morphometric Analysis

See the online supplement for additional details on the methods used to make these measurements.

Peripheral Blood Leukocyte and Lung Cell Isolation

See the online supplement for additional details on the methods used to make these measurements.

Flow Cytometric Analysis

To minimize nonspecific binding cells were first incubated with FcBlock (anti-CD16/CD32; BD PharMingen, Mississauga, ON, Canada) in FACS buffer (0.5% BSA in PBS) for 15min at 4°C. Antibodies were titrated to determine optimal concentration.

Gating strategies employed in the analysis of flow cytometric data are shown in Figure E1 of the *Online Data Supplement*. See the online data supplement for additional details on the methods used to make these measurements.

Splenocyte Culture

Splenocytes were isolated, resuspended in complete RPMI (RPMI supplemented with 10% FBS - Sigma-Aldrich; 1% L-glutamine; 1% penicillin/streptomycin – Invitrogen, Grand Island, NY, USA; and 0.1% β -mercaptoethanol – Invitrogen) at a concentration of 8×10^6 cells/ml and cultured in medium alone, or with medium supplemented with HDM (31.25 μ g/ml) in a flat-bottom, 96-well plate (Becton Dickinson), in triplicate. After 5 days of culture, supernatants were harvested and triplicates were pooled for cytokine measurements. See the online supplement for additional details on the methods used to make these measurements.

Cytokine and Immunoglobulin Measurements by ELISA

See the online supplement for additional details on the methods used to make these measurements.

Airway Responsiveness Measurements

See the online supplement for additional details on the methods used to make these measurements.

Data Analysis

Data were analyzed using SigmaStat v2.03 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean \pm SEM. Results were analyzed using *t* test, or one or two way analysis of variance (ANOVA) with Tukey's *post hoc* test. If a data set failed the normality test, appropriate transformations were applied and the transformed data set was re-analyzed. Differences were considered statistically significant when *P* values were less than < 0.05 .

RESULTS

Impact of anti-CCR3 treatment on eosinophil kinetics and HDM-induced remodeling:

To directly investigate the requirement for eosinophils in the development of allergic remodeling induced by HDM exposure we first utilized a rat monoclonal anti-mouse CCR3 Ab (α CCR3) to selectively deplete eosinophils. The binding and depleting activity of this Ab have been previously shown to be specific for eosinophils (18). Moreover, it has been successfully utilized to completely ablate eosinophils in acute OVA-induced models of allergic airway disease (19, 20). Preliminary studies were conducted to determine an optimal dosing regimen that would yield the greatest depletion over a 4 week course of HDM exposure without eliciting adverse effects (data not shown). Separate groups of BALB/c mice were exposed to saline or HDM i.n., daily 5 days/week for up to 4 weeks and HDM-exposed groups were concurrently treated with 0.5mg/dose of α CCR3 or a control Ab (IgG). Mice were sacrificed following 2, 3, and 4 weeks of exposure and eosinophil kinetics were examined in the blood, lung tissue and BAL in

order to track depletion throughout the study. We observed that α CCR3 treatment dramatically reduced blood eosinophils to levels that were below baseline (Figure 1A). Furthermore, α CCR3 markedly decreased the extent of eosinophilia in the airway and lung tissue compartments to levels that were at or near baseline (Figures 1B–C). Based on calculations of area under the curves, we estimate that the extent of overall depletion was on the order of 80–85% in the BAL/lung over the course of the experiment (*see* Table E1 in the online data supplement). We further analyzed whole lung cells for the presence of Th2 cells by flow cytometry. Importantly, α CCR3 treatment and the resulting decrease in eosinophilia had no impact on the extent of lung Th2 cells (Figure 1D). Notably, differential cell count analysis revealed that the number of mononuclear cells and neutrophils in the BAL at 4 weeks was also unaffected (Figure E2).

We assessed the impact of eosinophil ablation on HDM-induced remodeling by examining subepithelial collagen deposition, smooth-muscle thickness and goblet cell hyperplasia/mucus production. We observed that both HDM-exposed IgG and α CCR3 treated groups developed significant increases in all three remodeling-associated parameters compared to mice exposed to saline alone (Figure 2). Interestingly, α CCR3 treated HDM-exposed mice developed increases in collagen deposition, smooth muscle and goblet cells/mucus production that were identical to HDM IgG-treated mice (Figure 2).

Impact of eosinophil deletion on HDM-induced inflammation, immunity and remodeling in Δ dblGATA mice.

To confirm the results obtained from the α CCR3 studies and exclude the possibility that the residual population of eosinophils in the lung/airway of α CCR3 treated mice, however small in number, was sufficient to drive the remodeling response we subjected Δ dblGATA eosinophil-deficient mice to 5 weeks of HDM exposure. We observed that both WT and eosinophil-deficient HDM-exposed mice developed robust airway inflammation as evidenced by significant increases in the BAL total cell number compared to their corresponding saline-exposed controls (Figure 3A). However, the increase in total cells observed in the GATA mice was significantly lower (~35% fewer cells) than that observed in WT animals. Further examination of the BAL showed that this difference was largely due to the absence of eosinophils with only a minor decrease in mononuclear cells (~23% fewer mononuclear cells) and no change in neutrophils being observed in the GATA compared to WT HDM-exposed mice (Figures 3B-3C and data not shown). Consistent with the BAL data, no differences in lung neutrophils were noted between the two HDM-exposed groups (data not shown) and no eosinophils were detected in the lungs of HDM-exposed GATA mice by flow cytometry and differential cell count analysis (Figure 3D and data not shown). We also examined whether the absence of eosinophils had any effect on the T cell component of the lung inflammatory infiltrate. To this end, we documented significant increases in CD69⁺CD4⁺ (activated) and T1/ST2⁺ CD4⁺ (Th2) T cells in the lungs of HDM-exposed GATA eosinophil-deficient and WT mice relative to their saline-exposed controls (Figures 3E-3F). However, while

the increases in activated T cells were comparable between HDM-exposed GATA and WT mice, we noted a slight reduction in the number of lung Th2 cells in the GATA group.

Next, we ascertained whether the absence of eosinophils had any impact on B and/or T cell function. We evaluated serum levels of HDM-specific IgE in WT and GATA HDM-exposed mice and noted that the levels of IgE were comparably increased (Figure 4A). In addition, splenocytes harvested from HDM-exposed GATA mice produced equal amounts of IL-5 and IL-13, following *in vitro* stimulation with HDM, as those from HDM-exposed WT mice (Figure 4B).

Qualitative assessment of airway remodeling by histologic examination of the lung tissues suggested that the remodeling response developed to the same extent in GATA and WT mice following 5 weeks of HDM exposure (Figure 5). Indeed, morphometric analysis demonstrated similar increases in subepithelial collagen deposition, smooth muscle thickness and goblet cells/mucus production between HDM-exposed GATA and WT groups (Figure 6). Importantly, the similarity in these responses was maintained when the data were expressed as fold increase over baseline (i.e. over the corresponding saline-exposed control group).

Finally, we examined the effect of eosinophil deletion on lung function by evaluating total respiratory resistance responses to increasing doses of methacholine (MCh) in WT and GATA HDM-exposed mice (Figures 7A and E3). We observed that both

HDM-exposed WT and GATA mice displayed marked increases in maximum resistance and airway reactivity compared to their corresponding saline-exposed controls (Figures 7B-C). However, a small statistically significant ($P = 0.049$) decrease in maximum resistance and a non-significant ($P = 0.089$) trend towards decreased airway reactivity were noted in the GATA HDM-exposed group versus WT HDM mice (Figures 7B-C).

Impact of eosinophil deletion on HDM-induced inflammation and remodeling in PHIL mice.

In light of the reported differences pertaining to the role of eosinophils in the generation of OVA-induced allergic airway inflammation and hyperresponsiveness in Δ dblGATA versus *PHIL* eosinophil-deficient mice (11, 17, 21) we also explored the effects of eosinophil deletion on HDM-induced responses in the *PHIL* strain of mice. We exposed *PHIL* eosinophil-deficient mice and their WT-littermate controls to HDM for 5 weeks. Similar to GATA mice, *PHIL* mice developed significant airway inflammation in the BAL in response to 5 weeks of HDM exposure, compared to saline-exposed *PHIL* controls, although this response was markedly lower (~57% fewer cells) than that observed in WT HDM-exposed littermate controls (Figure 8A). However, unlike what we observed in the GATA mice, the decrease in BAL inflammation in the *PHIL* mice was due not only to the absence of eosinophils but also to considerable reductions in the numbers of mononuclear cells and neutrophils (~43% and ~40% fewer, respectively, relative to HDM-exposed WT mice; Figures 8B-D). Interestingly, although eosinophils were virtually absent from the lung inflammatory infiltrate we did not detect any decreases in the number of lung Th2 cells in *PHIL* HDM-exposed mice (Figures 8E-F). Equivalent

increases in the levels of serum HDM-specific IgE were also observed between HDM-exposed *PHIL* and WT groups (Figure E4).

We proceeded to investigate the consequence of eosinophil deletion in *PHIL* mice on the generation of HDM-induced remodeling. Although airway remodeling was clearly evident in HDM-exposed *PHIL* eosinophil-deficient mice, modest decreases in peribronchial collagen deposition and smooth muscle thickness were observed relative to HDM-exposed WT mice (Figures 9A–B). No differences in goblet cells/mucus production were noted between *PHIL* and WT mice following 5 weeks of HDM (Figure 9C).

DISCUSSION:

It has become evident that eosinophils can be involved in an array of biological processes including host defense, antigen presentation, T cell recruitment, leukocyte activation and survival, and tissue repair (22–26). From this perspective, eosinophils could contribute to various facets of the allergic response. However, the extent to which they participate in the above processes or in disease pathogenesis in general has been, in many cases, controversial (24, 27–29). That eosinophils can produce a number of pro-reparative/fibrotic mediators and are often seen to be intimately associated with the airway mucosa in allergic asthmatics has spurred the proposition that eosinophils are critically involved in the development of allergic airway remodeling.

Numerous experimental studies have investigated the involvement of eosinophils in the remodeling response. By far, the most common approach employed to abolish eosinophilia has involved interference with IL-5 function. Of at least 6 studies using an IL-5 based approach some have shown that, in the absence of eosinophils, OVA-induced remodeling is almost entirely abrogated (5, 10), while others observed only partial decreases (6, 8) or absolutely no impact of eosinophil ablation on remodeling (7, 9). The reasons for these discrepancies are not immediately apparent but differences in the strain of mouse, protocols employed or approaches taken to eliminate eosinophils have been proposed as plausible explanations. However, it should be noted that even in instances where the same experimental protocols and strain of mice were used to investigate the requirement of eosinophils in remodeling, contradictory results were reported (7, 8). Conversely, studies employing differing methods of eosinophil ablation, strains of mice and experimental protocols have reported similar findings regarding the involvement of eosinophils in remodeling (5, 6, 10). The contribution of eosinophils to the development of allergic remodeling has also been examined, more directly, using the Δ dblGATA strain of eosinophil-deficient mice (11). Interestingly, GATA eosinophil-deficient mice subjected to chronic OVA exposure showed considerably reduced levels of total lung collagen as well as decreased numbers of total and proliferating smooth muscle cells, demonstrating that eosinophils did significantly contribute to remodeling in that experimental system. Collectively, these findings have fueled, in spite of the notable discrepancies, the general notion that eosinophils play a significant role in allergic airway remodeling.

Importantly, virtually all of the experimental findings discussed above have been conducted in the context of OVA-based models of allergic airway disease. Although these models have been instrumental to our understanding of disease pathogenesis, it is becoming increasingly evident that at least some of the underlying mechanisms that govern allergic airway inflammation and remodeling in those models differ from those induced by exposure to a CEA. For example, while TGF- β has been shown to be a critical mediator of OVA-induced remodeling (12-14) we have previously demonstrated that TGF- β is not required for the generation of remodeling induced by respiratory mucosal exposure to HDM (15), one of the most pervasive aeroallergens worldwide. Therefore, in the current study we explored the role of eosinophils in HDM-induced remodeling and adaptive immunity. We employed two distinct, but complementary, strategies to specifically eliminate eosinophils: 1) an α CCR3 eosinophil depleting Ab, a valuable alternative to genetically-based strategies, and 2) two separate lines of eosinophil-deficient mice. Using the α CCR3 Ab we were able to deplete approximately 80-85% of BAL/lung eosinophils over the course of a 4 week HDM exposure protocol. Although we did not achieve 100% depletion, eosinophils were reduced by a considerable degree; especially when the duration and intensity of the inflammatory response elicited by continuous HDM-exposure is taken into account. Interestingly, we observed that this decrease in eosinophils had absolutely no impact on the development of HDM-induced remodeling as we noted increases in subepithelial collagen deposition, smooth muscle thickening and mucus production indistinguishable to those observed in HDM-exposed mice treated with a control Ab. Along the same lines, we also observed that remodeling

developed, undiminished, in Δ dblGATA eosinophil-deficient mice following 5 weeks of HDM exposure. Additionally, goblet cell hyperplasia and mucus production were completely unaffected in HDM-exposed *PHIL* eosinophil-deficient mice. Moreover, although we documented significant decreases in collagen deposition and smooth muscle thickness in these mice compared to their WT HDM-exposed littermates, both collagen and smooth muscle were increased in the *PHIL* HDM-exposed group relative to their saline-exposed controls. Of particular significance in the case of the *PHIL* mice, the decreases in remodeling were associated not only with the absence of eosinophils but also with considerable reductions in the numbers of airway mononuclear cells and neutrophils, making it difficult to ascribe a direct role for eosinophils in the decreased remodeling response. Together, these data demonstrate that eosinophils are largely, if not entirely, dispensable for the development of HDM-induced remodeling.

The differential involvement of eosinophils on remodeling in OVA- versus HDM-based models provides further evidence of mechanistic differences, underlying the development of remodeling and, likely, other processes between these two systems. There are a number of reasons that could account for this, although the two most evident and, perhaps, most essential are the methods by which sensitization is achieved and the nature of the eliciting agents. In the case of OVA-based models, sensitization is most often accomplished by injecting purified OVA (typically >98% pure) into the peritoneum in combination with a chemical adjuvant (usually aluminium hydroxide or alum). These manipulations are necessary as OVA is an innocuous protein that when delivered to the lung on its own results in inhalation tolerance, not allergic disease (30, 31). In contrast,

sensitization to HDM is achieved via the respiratory mucosa in the absence of any exogenous adjuvant. Thus, the incipient targets of mucosally delivered HDM are, in all likelihood, different than those reached by OVA administered i.p. along with an adjuvant. Moreover, HDM extracts, unlike OVA, contain a vast array of protein and non-protein components of which many are intrinsically able to trigger responses, immune and otherwise, that favour the generation of allergic sensitization (32-39). We surmise that this ability, in conjunction with the site of sensitization, enables HDM with the capacity to elicit a complex network of molecular signatures and mucosal immune responses fundamentally distinct than those elicited by OVA-based systems. Indeed, whereas uric acid/NALP3 inflammasome/IL-1 β -mediated mechanisms are thought to drive responses induced in conventional OVA-based systems (40, 41), MD-2/TLR-4/GM-CSF/TSLP-dependent mechanisms have been shown to be critical to the induction of HDM-induced responses (34, 38, 42). By extension, the inherent diversity among aeroallergens (such as roaches, fungi and pollens) suggests that, at least until proven otherwise, dissimilar responses could be expected from these as well. An important implication that arises from this premise is that the responses elicited in OVA-based systems are, at least in part, misleading, not only from the perspective of our understanding of disease pathogenesis but also regarding our expectations of the therapeutic strategies that have been evaluated in them.

We also investigated the contribution of eosinophils to HDM-induced inflammation and immunity. Notably, the substantial reduction in the number of

BAL/lung eosinophils in HDM-exposed α CCR3 treated mice had no impact on pulmonary inflammation, nor did it affect markers of HDM-specific Th2-sensitization (data not shown). Similarly, GATA eosinophil-deficient mice mounted a robust Th2-polarized immune inflammatory response, following 5 weeks of HDM exposure. However, in addition to the absence of eosinophils small decreases in BAL mononuclear and lung Th2 cells were noted in these mice. These findings indicate that eosinophils are involved in HDM-induced inflammation, although their contribution appears to be auxiliary rather critical in nature. Furthermore, when considered in combination with our observations in the α CCR3 studies, the data suggest that even a small degree of eosinophilia is sufficient to mediate this enhancement. Interestingly, although *PHIL* eosinophil-deficient mice displayed marked increases in BAL/lung inflammation (relative to saline controls) following HDM exposure, we documented considerable reductions in BAL mononuclear cells and neutrophils, indicating that in these mice the absence of eosinophils was associated with a substantial decrease in the magnitude of the airway inflammatory response. That HDM-exposed *PHIL* mice had similar levels of HDM-specific IgE and numbers of lung Th2 cells as WT controls indicates that this may have been due to decreased cellular recruitment rather than impaired induction of adaptive immunity. It is unclear why decreases of such magnitudes were observed in *PHIL* but not in α CCR3-treated or GATA HDM-exposed mice but the similarity of the findings among the latter two groups, along with the fact that all of these experiments were done in the same background strain of mice (BALB/c) and utilized the same exposure protocol, suggests that this observation may be, in some part, an idiosyncratic feature of the *PHIL*

manipulation. Nonetheless, our data clearly demonstrate that eosinophils play little to no role in the establishment of HDM-induced allergic sensitization and a modest, augmentative, role in the generation of HDM-induced airway inflammation.

Our findings concerning the negligible role of eosinophils in the development of an immune adaptive Th2 response are in agreement with those documented by Humbles *et al.* who observed undiminished OVA-specific Th2-sensitization, airway inflammation and lung Th2 cells in GATA eosinophil-deficient mice compared to WT controls (11). In sharp contrast, Jacobsen *et al.* reported that *PHIL* eosinophil-deficient mice subjected to an acute OVA-exposure model displayed dramatic reductions in BAL and lung lymphocytes (21). Moreover, they reported that splenocytes harvested from *PHIL* sensitized and challenged mice produced markedly less IL-13 following OVA stimulation *in vitro* leading the authors to suggest that eosinophils play an essential part in establishing Th2-responses and allergic inflammation. The discrepancy between the Humbles and Jacobsen reports, both of which utilized very similar OVA-based protocols, has recently been attributed, in large part, to differences in background strain. Walsh *et al.* backcrossed the Δ dblGATA mutation (employed in the Humbles study; originally on the BALB/c background) onto the C57BL/6 background (the background strain used in the *PHIL* Jacobsen study) and subjected these mice to an acute OVA exposure protocol (43). The authors observed that WT and BALB/c Δ dblGATA OVA-exposed mice developed similarly robust allergic inflammation, whereas the C57BL/6 Δ dblGATA OVA-exposed mice displayed a marked reduction in this response. However, C57BL/6 Δ dblGATA OVA-exposed mice had

similar serum levels of OVA-specific IgE as C57BL/6 WT mice and splenic T cells from these mice responded equally well to OVA restimulation *in vitro* as T cells from WT controls, suggesting that the reduction in lung inflammation, in the absence of eosinophils, could be due to defects in cellular recruitment and not impaired sensitization. Thus, these findings do not explain the observation that *PHIL* mice show diminished sensitization on account of their C57BL/6 background. The findings of Walsh *et al.* further imply that at least some component of the decreased immune responsiveness observed in C57BL/6 *PHIL* mice may be a distinctive attribute of this specific mouse line. At variance with the notion that eosinophils are important mediators of allergic inflammation in C57BL/6 mice at all is an earlier report by Justice *et al.* showing that eosinophil ablation during only the challenge phase of their exposure protocol (via α CCR3 Ab treatment) had no impact on the influx of other leukocytes, including lymphocytes, to the airways (19).

Interestingly, Fulkerson *et al.* reported in a model of *Aspergillus fumigatus*-induced allergic airway disease that, in contrast to the findings by Humbles *et al.* in an OVA-based system and somewhat unlike the data presented here in an HDM-system, the number of airway lymphocytes was greatly reduced (~50%) in *aspergillus*-exposed Δ dblGATA BALB/c mice (44). This finding, in conjunction with those above, suggests that the nature of the allergen may also play a prominent role in determining the extent to which eosinophils contribute to allergic inflammation. We note that sensitization (i.e. Ag-specific Ig levels) was unaffected in *aspergillus*-exposed eosinophil-deficient mice. Nor was it affected in another study where eosinophils were eliminated by interfering with IL-5,

even when airway/lung mononuclear cells were decreased (45). Therefore, while the requirement for eosinophils in the development of allergic inflammation may ultimately depend on the strain of mouse and the nature of the allergen employed it does not appear as though eosinophils are critically required for the generation of allergic sensitization, under any conditions.

Lastly, physiological evaluation of lung function in GATA HDM-exposed mice revealed that eosinophils contributed in a minor way, at best, to the development of HDM-induced airway hyperresponsiveness. Historically, the literature pertaining to the impact of eosinophils on airway responsiveness, in experimental models, has been highly conflicted. Of the nearly two-dozen studies that have been conducted, approximately half have reported that eosinophils do play a role in this process and half not (reviewed in 46). Importantly, the functional consequence of eosinophils in human allergic asthma has been investigated in several clinical trials. Collectively, the results of these studies suggest that eosinophils play only a marginal role in airway hyperresponsiveness and lung dysfunction in, at least, the overall population of allergic asthmatics (47-51).

A considerable body of experimental research, largely in OVA-based models, has instigated the notion that eosinophils are principal mediators of allergic inflammation and airway remodeling. In the present study, we have demonstrated that eosinophils are dispensable for the development of adaptive Th2 immunity and airway remodeling induced by the prolonged exposure to house dust mite. Rather than fostering a futile dichotomy (OVA versus HDM), our findings highlight a simple and perhaps

underappreciated concept: that the role of a given cell, or molecule for that matter, is contextual and, therefore, that great caution must be applied to not exert unqualified universal forecasts. From this perspective, the divergence of findings regarding the role of eosinophils on airway inflammation, Th2 immunity and airway remodeling in systems using OVA and CEAs, such as HDM or fungal antigens, may not be contradictory but, rather, an expression of the heterogeneity that underlies allergic responses. In this regard, it has now become clear that allergic airway diseases encompass an array of distinct phenotypes (52, 53), or “endotypes” as referred to by G. Anderson (52). Thus, the diversity of responses observed in experimental systems may be a consonant, but surely fractional, fitting to the heterogeneity of human asthma. Yet, an unselective attachment to experimental simplicity and habitual practices may alienate experimental research from the complexity of human biological processes and foster deceiving interpretations with respect to both disease pathogenesis and therapeutic expectations.

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FIGURE LEGENDS:

Figure 1. Systemic and local impact of anti-CCR3 (α CCR3) treatment on eosinophils and Th2 cells in house dust mite (HDM)-exposed mice. Separate groups of mice were exposed to house dust mite (HDM) extract or saline for up to 4 weeks. HDM-exposed mice were concurrently treated, on days 3, 6, 9, 13, 16, 19, 22, and 25 with either an α CCR3 eosinophil-depleting antibody (Ab) or a control IgG Ab (0.5mg/dose). Different groups of mice were sacrificed 24 hours after the second and third weeks of exposure or 72 hours after the last HDM/saline exposure (i.e. fourth week). Percentage of eosinophils (Eos) in (A) the blood and (B) the bronchoalveolar lavage (BAL), based on differential cell count analysis. Percentage of (C) Eos ($CD45^+ Gr-1^{low/int} SSC^{int/high}$) and (D) Th2 cells ($CD45^+ CD3^+ CD4^+ T1/ST2^+$) in the lung based on flow cytometric analysis of whole lung digest using the gating strategies described in Figure E1 of the *Online Supplement*. n = 2-3/group in (A-D) for the 2 and 3 week time points; n = 5-7/group in (A) and n = 6-7/group in (B-D) for the 4 week time point. Data are expressed as mean \pm SEM, where possible. P<0.05 compared with the *saline-exposed group and ^SHDM-exposed α CCR3-treated group, respectively. Analysis performed on the 4 week time point only.

Figure 2. Impact of anti-CCR3 (α CCR3) treatment on house dust mite (HDM)-induced remodeling. Separate groups of mice were exposed to HDM (*black bars*) or saline (*white bars*) for 4 weeks. HDM-exposed mice were concurrently treated, on days 3, 6, 9, 13, 16, 19, 22, and 25 with either α CCR3 or control IgG (0.5mg/dose) antibody (Ab). Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung

tissue obtained 72 hours after the last HDM/saline exposure: (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition, (B) immunohistochemistry for α -smooth muscle actin (α -SMA) indicating contractile elements in the airway wall (*brown*; *insets* show non-specific staining in the corresponding negative control section) and (C) periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells (*magenta*; *insets* show colour inverted image used for morphometric analysis). (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR, α -SMA or PAS. All pictures were taken at 20x magnification except *insets* in (C) which were at 40x; n = 5-7/group. Data are expressed as mean \pm SEM and are from one of two independent experiments that yielded similar results. *P<0.05 compared with the saline-exposed group.

Figure 3. Impact of eosinophil deletion on house dust mite (HDM)-induced allergic airway inflammation in Δ dblGATA mice. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM (*black bars*) or saline (*white bars*) for 5 weeks and sacrificed 72 hours after the last HDM exposure. (A-C) Total and differential cell count analysis showing the number of total cells (TCN), mononuclear cells (MN), and eosinophils (Eos) in the bronchoalveolar lavage (BAL). (D-F) Flow cytometric analysis of whole lung digests showing the number of Eos (CD45⁺ Gr-1^{low/int} SSC^{int/high}), activated T cells (CD45⁺ CD3⁺ CD4⁺ CD69⁺) and Th2 cells (CD45⁺ CD3⁺ CD4⁺ T1/ST2⁺); gating strategies are described in Figure E1 of the *Online Supplement*. n = 4-8/group. Data are expressed as mean \pm SEM and are from one of two independent experiments that yielded

similar results. $P < 0.05$ compared with the *corresponding saline-exposed group and Δ dblGATA-exposed Δ dblGATA group, respectively.

Figure 4. Impact of eosinophils on house dust mite (HDM)-specific Th2-associated adaptive immune responses. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM or saline for 5 weeks and sacrificed 72 hours after the last exposure. (A) HDM-specific Th2-associated IgE levels in the serum. (B) Splenocyte cytokine production; splenocytes from individual mice were cultured in medium alone (*white bars*) or stimulated with HDM (*black bars*) *in vitro*. $n = 5-8$ /group in (A) and $n = 4-7$ /group in (B). Data are expressed as mean \pm SEM and are from one of two independent experiments that yielded similar results. $P < 0.05$ compared with the *corresponding saline-exposed group.

Figure 5. Histological evaluation of house dust mite (HDM)-induced allergic remodeling in Δ dblGATA mice. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM or saline for 5 weeks. Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure: (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition, (B) immunohistochemistry for α -smooth muscle actin (α -SMA) indicating contractile elements in the airway wall (*brown*; *insets* show non-specific staining in the corresponding negative control section) and (C) periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells

(magenta; insets show colour inverted image used for morphometric analysis). All pictures were taken at 20x magnification except insets in (C) which were at 40x.

Figure 6. Morphometric analysis of the impact of eosinophil deletion on house dust mite (HDM)-induced remodeling in Δ dblGATA mice. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM (black bars) or saline (white bars) for 5 weeks. Lung tissues were obtained 72 hours after the last HDM exposure and cross-sections were cut and stained with Picro Sirius red (PSR), α -smooth muscle actin (α -SMA) or periodic acid-Schiff (PAS). Morphometric analysis of lung histology was performed. Data represent the percentage of the area of interest that is stained with (A) PSR – indicating subepithelial collagen deposition, (B) α -SMA – indicating smooth muscle thickness and (C) PAS – indicating mucus production by epithelial goblet cells. Inset graphs express data as the fold increase over the corresponding saline-exposed group. n = 5-8/group. Data are expressed as mean \pm SEM. P<0.05 compared with the *corresponding saline-exposed group and [†]HDM-exposed WT group, respectively.

Figure 7. Impact of eosinophil deletion on house dust mite (HDM)-induced airway responsiveness to methacholine (MCh) in Δ dblGATA eosinophil-deficient mice. Separate groups of wild-type (WT; circles) and Δ dblGATA (triangles) mice were exposed to HDM (black symbols/bars) or saline (white symbols/bars) for 5 weeks. (A) Total respiratory resistance (R) was measured at increasing doses of MCh. Data show the peak respiratory resistance measured after saline and each of the MCh challenges. Complete dose response

data are shown in Figure E3 of the *online supplement*. (B) Maximum respiratory resistance. (C) Airway reactivity, measured by the slope of the linear regression between peak respiratory resistance and the \log_{10} of the MCh dose using the data from the 3.125, 12.5, and 50 mg/ml doses. Measurements in (B and C) are expressed as absolute values and as the fold increase over the corresponding saline-exposed group. $n = 6-7/\text{group}$ in (A and B) and $5-7/\text{group}$ in (C). Data are expressed as mean \pm SEM and are from one of three independent experiments that yielded similar results. $P < 0.05$ compared with the *corresponding saline-exposed group and § HDM-exposed $\Delta\text{dblGATA}$ group, respectively.

Figure 8. Impact of eosinophil deletion on house dust mite (HDM)-induced allergic airway inflammation in *PHIL* mice. Separate groups of wild-type (WT) littermate control and *PHIL* eosinophil-deficient mice were exposed to HDM (*black bars*) or saline (*white bars*) for 5 weeks and sacrificed 72 hours after the last exposure. (A-D) Total and differential cell count analysis showing the number of total cells (TCN), mononuclear cells (MN), neutrophils (Neut), and eosinophils (Eos) in the bronchoalveolar lavage (BAL). (E-F) Flow cytometric analysis of whole lung digests showing the number of Eos ($\text{CD45}^+ \text{Gr-1}^{\text{low/int}} \text{SSC}^{\text{int/high}}$) and Th2 cells ($\text{CD45}^+ \text{CD3}^+ \text{CD4}^+ \text{T1/ST2}^+$); gating strategies are described in Figure E1 of the *Online Supplement*. $n = 3-6/\text{group}$ in (A-D) and $3-5/\text{group}$ in (E-F). Data are expressed as mean \pm SEM. $P < 0.05$ compared with the *corresponding saline-exposed group and § HDM-exposed *PHIL* group, respectively.

Figure 9. Morphometric analysis of the impact of eosinophil deletion on house dust mite (HDM)-induced remodeling in *PHIL* mice. Separate groups of wild-type (WT) littermate control and *PHIL* eosinophil-deficient mice were exposed to HDM (*black bars*) or saline (*white bars*) for 5 weeks. Lung tissues were obtained 72 hours after the last HDM exposure and cross-sections were cut and stained with Picro Sirius red (PSR), α -smooth muscle actin (α -SMA) or periodic acid-Schiff (PAS). Morphometric analysis of lung histology was performed. Data represent the percentage of the area of interest that is stained with (A) PSR – indicating subepithelial collagen deposition, (B) α -SMA – indicating smooth muscle thickness and (C) PAS – indicating mucus production by epithelial goblet cells. *Inset* graphs express data as the fold increase over the corresponding saline exposed group. n = 3-6/group. Data are expressed as mean \pm SEM. P<0.05 compared with the *corresponding saline-exposed group and ^SHDM-exposed *PHIL* group, respectively.

Figure 1

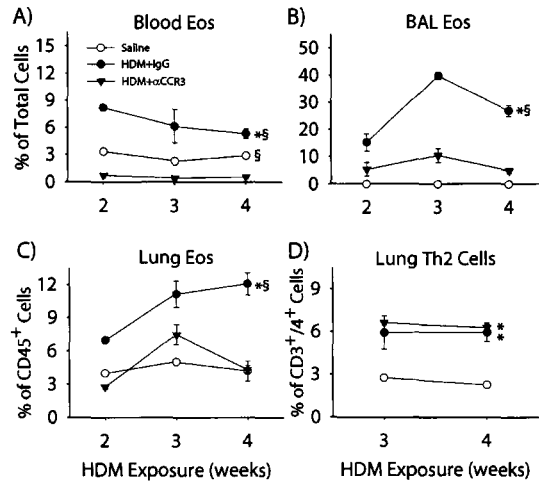


Figure 2

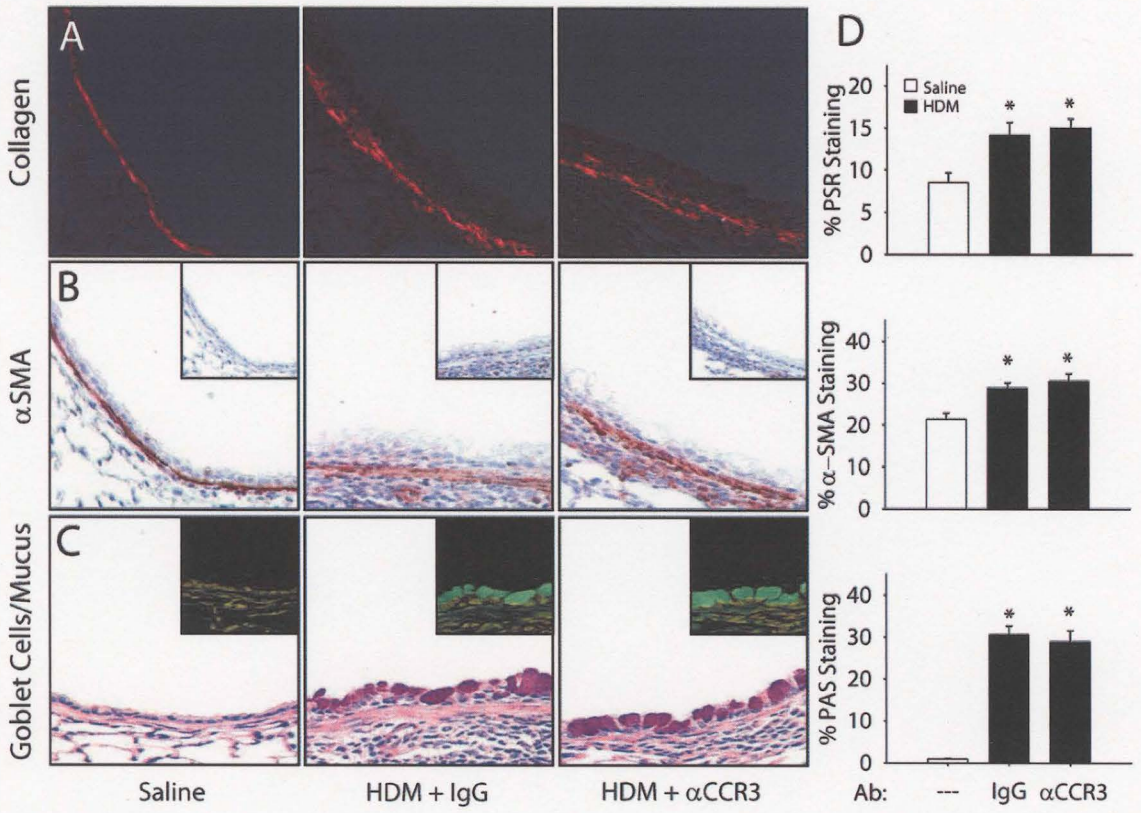


Figure 3

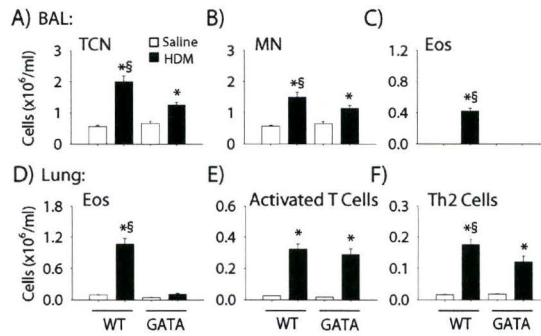


Figure 4

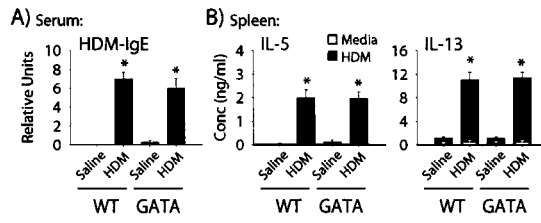


Figure 5

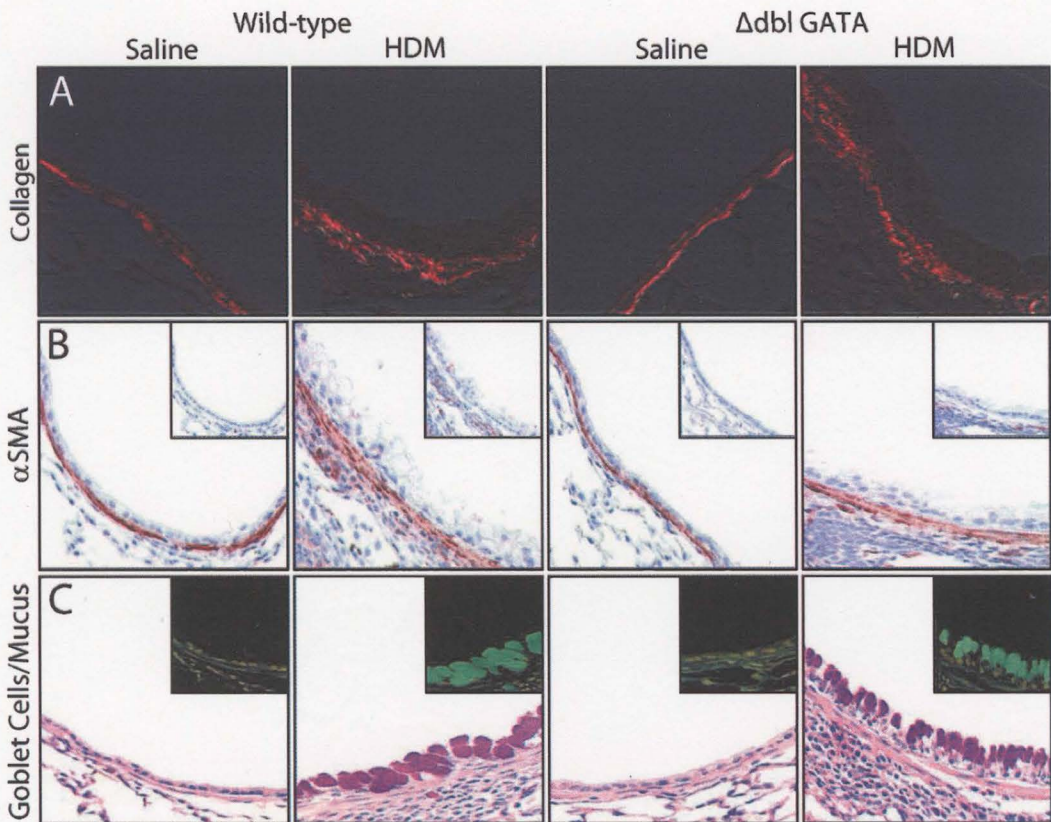


Figure 6

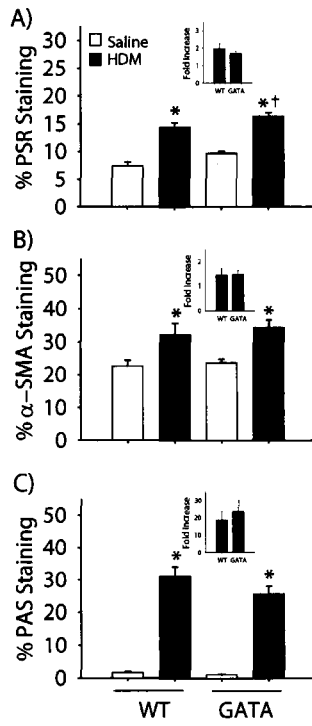


Figure 7

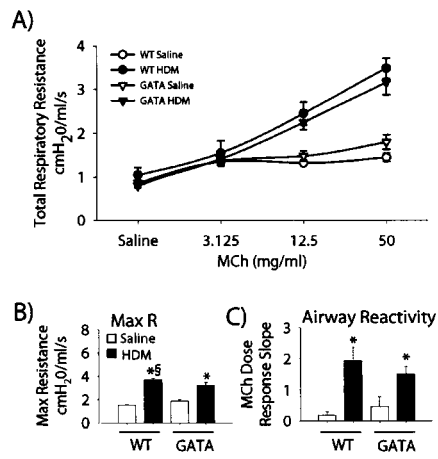


Figure 8

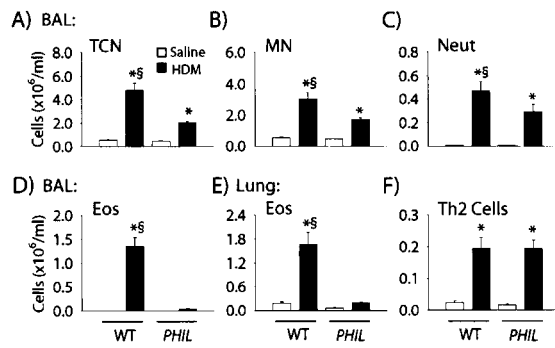
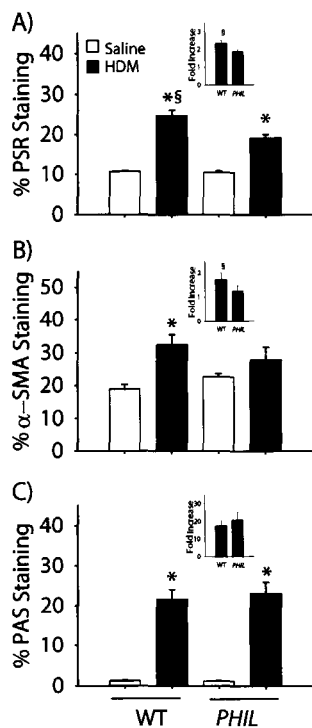


Figure 9



Online Data Supplement

Eosinophils are Dispensable for House Dust Mite-Induced Allergic Airway Remodeling and Immunity

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METHODS

Collection and Measurement of Specimens

Bronchoalveolar lavage (BAL), lungs, blood and spleen were collected approximately 72 hours after the last HDM challenge. BAL was performed as previously described (E1). Briefly, lungs were dissected and the trachea cannulated with a polyethylene tube (outer/inner diameter = 0.965/0.58mm; Becton Dickinson, Sparks, MD, USA). Lungs were lavaged twice with cool phosphate-buffered saline (PBS; 0.25ml followed by 0.2ml) and approximately 0.25–0.35ml of the instilled fluid was consistently retrieved. Total cell counts were determined using a hemocytometer. Each BAL sample was then centrifuged and supernatants collected and stored at -20°C . Cell pellets were subsequently resuspended in PBS and smears were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, PA, USA) at 300rpm for 2min. Protocol Hema 3 stain set (Fisher Scientific, Toronto, ON, Canada) was used to stain all smears. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as eosinophils, mononuclear cells or neutrophils.

Morphometric Analysis

Analysis of lung sections stained for PSR, PAS or α -SMA were performed as previously described in (E2). Briefly, multiple images were taken of the major airway from each of the sections of each individual mouse lung, being careful to avoid regions of the airway that were adjacent to a blood vessel. Approximately 3–8 images were taken at 20x magnification for PSR and α -SMA analysis and 5–12 for PAS analysis. Images were

captured with OpenLab software (Improvision, Guelph, ON, Canada) via a Leica camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada). Image analysis was performed using a custom computerized analysis system (Northern Eclipse software version 7; Empix Imaging, Mississauga, ON, Canada). For quantification of PSR staining a line is drawn along the base of the epithelium and a second parallel line is projected 40 μ m away by the computer, towards the tissue, creating a 'region of interest'. In the case of PAS and α -SMA analyses the user drawn line is made along the interior side of the smooth muscle layer or epithelium, respectively, and the second line is projected in the direction of the airway lumen. Distances of 20 and 35 μ m were used for α -SMA and PAS analysis respectively. Within these 'regions of interest', a colour range is selected that best captures the stain of interest and minimizes or eliminates inclusion of background staining. The total area of the 'region of interest' and the stained area are quantified and weighted averages are calculated for each individual mouse. The data shown represent the average percentage of the area of interest that is stained with PSR, α -SMA or PAS. In the specific case of α -SMA analysis some non-specific binding of the primary Ab is commonly observed as both the Ab and tissue are from the same species (i.e. mouse). Generally, non-specific staining does not interfere with quantitative analysis. Regardless, negative controls are generated for each individual tissue section and any non-specific staining in the 'region of interest' is taken into account during morphometric analysis.

Peripheral Blood Leukocyte and Lung Cell Isolation

PBLs were isolated by resuspending blood in ACK lysis buffer (0.5 M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA , pH 7.2-7.4) at a 1:10 volume ratio for 5min at room temperature. Lysis was neutralized with FACS buffer (0.5% BSA in PBS) supplemented with 1mM EDTA. Cells were centrifuged at 1500rpm for 5 min at 4°C and lysis was repeated. PBLs were then washed twice in the FACS buffer EDTA solution and cytopins were prepared, stained, and counted as described above for the BAL. Lungs were collected, cut into small pieces (~1mm in diameter using scissors) and agitated at 37°C for 1hr in 4ml of 150U/ml collagenase type III (Life Technologies Inc., Burlington, ON, Canada) in HBSS. Lung pieces were then triturated through a 40 μm cell strainer (BD), using the plunger end of a 3ml syringe, into FACS buffer. Cells were washed once with FACS buffer, resuspended, and filtered again prior to staining for flow cytometric analysis. Cytospins were also prepared from an aliquot of the lung cell suspension for differential cell counting as described above for the BAL.

Flow Cytometric Analysis

To minimize nonspecific binding cells were first incubated with FcBlock (anti-CD16/CD32; BD PharMingen, Mississauga, ON, Canada) in FACS buffer for 15min at 4°C. For each antibody combination, 2×10^6 cells were incubated with mAbs at 4°C for 30min. Cells were then washed twice with FACS buffer. Data were collected using a LSR II™ (BD) flow cytometer and analyzed using FlowJo™ software (Tree Star Inc., Ashland, OR, USA). The following antibodies were used: anti-CD4 (allophycocyanin-conjugated RM4-5 clone), anti-CD45 (allophycocyanin-Cy7-conjugated 30-F11 clone),

anti-CD69 (phycoerythrin-Cy7-conjugated H1.2F3 clone), anti-CD3 ϵ (pacific blue-conjugated 500A2 clone), anti-Gr-1 (phycoerythrin-Cy7-conjugated RB6-8C5 clone), anti-IgG₁ (fluorescein isothiocyanate-conjugated R3-34 clone), and anti-IgG_{2b} (phycoerythrin-Cy7-conjugated A95-1 clone) purchased from BD PharMingen; anti-T1/ST2 (fluorescein isothiocyanate-conjugated DJ8 clone) purchased from MD Biosciences (St. Paul, MN, USA) and anti-CD3 ϵ (phycoerythrin-Cy5.5-conjugated 145-2C11 clone) and anti-GR-1 (phycoerythrin-Cy5.5-conjugated RB6 8C5 clone) purchased from ebioscience (San Diego, CA, USA). Isotype controls were used for T1/ST2 and Gr-1 and a fluorescence minus one control was used for CD69. Antibodies were titrated to determine optimal concentration. Gating strategies employed in the analysis of flow cytometric data are shown in Figure E1 of the *Online Supplement*.

Splenocyte Culture

Splenocytes were isolated by triturating whole spleens through a 40 μ m cell strainer (BD), using the plunger from a 3ml syringe, into HBSS. Cells were then centrifuged at 1200rpm for 10min at 4°C and red blood cells were lysed using ACK lysis buffer. HBSS was used to stop lysis after 1min (1:10 volume ratio). Splenocytes were then washed in complete RPMI (RPMI supplemented with 10% FBS – Sigma-Aldrich; 1% L-glutamine; 1% penicillin/streptomycin – Invitrogen, Grand Island, NY, USA; and 0.1% β -mercaptoethanol – Invitrogen). Splenocytes were then resuspended in complete RPMI at a concentration of 8x10⁶ cells/ml and cultured in medium alone, or with medium supplemented with HDM (31.25 μ g/ml) in a flat-bottom, 96-well plate (BD), in triplicate; 100 μ l of medium \pm HDM + 100 μ l of cell suspension per well. After 5 days of culture,

supernatants were harvested and triplicates were pooled and stored at -20°C for cytokine measurements.

Cytokine and Immunoglobulin Measurements by ELISA

Levels of mouse interleukin (IL)-5 and IL-13 were measured by ELISA using DuoSet kits purchased from R&D Systems (Minneapolis, MN, USA) and performed according to the manufacturer's instructions. Each of these assays has a threshold of detection between 1.5–5pg/ml. Levels of HDM-specific IgE were measured by sandwich ELISA as previously described in (E3).

Airway Responsiveness Measurements

Mice were anesthetized with nebulized isoflurane (3% with 1L/min O_2), paralyzed with pancuronium bromide (50 $\mu\text{g}/\text{kg}$ i.p.) to prevent respiratory effort during measurement, tracheostomized with a blunted 18-gauge needle, and mechanically ventilated with a small animal computer-controlled piston ventilator (flexiVent, SCIREQ Inc.) (E4). The ventilator was set at 200breaths/min with a tidal volume of 0.25ml. The respiratory rate was slowed during nebulization to provide 5 large breaths of aerosol at a tidal volume of 0.8ml. A script for the automated collection of data was initiated, with a positive-end-expiratory pressure of 3cm of H_2O applied by submerging the expiratory line in water. Airway responsiveness was measured by total respiratory resistance (R) responses to nebulized saline and increasing doses of methacholine (MCh; 3.125, 12.5, and 50mg/ml; Sigma-Aldrich). To provide a constant volume history, data collection (for saline and each dose of MCh) was preceded by two 6s inspiration to TLC perturbations. This was

followed by 2 baseline measurements and then the user was prompted to nebulize saline/MCh. For each dose, 12 “Quick-Prime-3” perturbations (3s in duration) were performed over an ~150s period. All perturbations were separated by a 10s interval. Total respiratory resistance was determined by fitting the data with the single compartment model of respiratory system mechanics. Model fits whose coefficient of determination was less than 0.8 were excluded from data analysis. Evaluation of airway responsiveness was based on the peak respiratory resistance measured in the ~150s after saline/MCh nebulizations. Airway reactivity was quantified by the slope of the linear regression between peak respiratory system resistance and the \log_{10} of the MCh dose, using the data from the 3.125, 12.5, and 50mg/ml doses only.

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FIGURE LEGENDS

Figure E1. Gating strategy employed in the analysis of flow cytometric data. Flow cytometric analysis of whole lung digests were performed as follows: whole lung leukocytes were first selected on the basis of CD45 expression, then ‘singlets’ were identified using FSC-W vs. FSC-A. Within this population neutrophils were identified as the Gr-1^{bright} SSC^{int} population (i.e. CD45⁺ Gr-1^{bright} SSC^{int}) and all other cells were gated and plotted on a graph of SSC vs FSC-A in order to identify eosinophils as the SSC^{int/high} population (i.e. CD45⁺ Gr-1^{low/int} SSC^{int/high}). We have previously shown that within this population >90% of the cells are eosinophils (E5). Activated (CD69⁺) and type-2 (T1/ST2⁺) Th cells were identified by plotting ‘singlets’ on a graph of SSC vs FSC-A, gating on the lymphocyte area, and subsequently gating on the CD3⁺ CD4⁺ population within the lymphocyte gate.

Figure E2. Impact of anti-CCR3 (α CCR3) treatment on airway inflammation in house dust mite (HDM)-exposed mice. Separate groups of mice were exposed to HDM (*black bars*) or saline (*white bars*) for 4 weeks. HDM-exposed mice were concurrently treated, on days 3, 6, 9, 13, 16, 19, 22, and 25 with either an α CCR3 eosinophil-depleting antibody (Ab) or a control IgG Ab (0.5mg/dose). Mice were sacrificed 72 hours after the last HDM/saline exposure. Differential cell count analysis showing (A) mononuclear cells (MN), (B) neutrophils (Neut) and (C) eosinophils (Eos) in the bronchoalveolar lavage. n = 6-7/group. Data are expressed as mean \pm SEM. P<0.05 compared with the *saline-exposed group and [§]HDM-exposed α CCR3 treated group, respectively.

Figure E3. Impact of eosinophil deletion on HDM-induced airway responsiveness to methacholine (MCh) in Δ dblGATA eosinophil-deficient mice. Separate groups of wild-type (WT; circles) and Δ dblGATA (triangles) mice were exposed to HDM (black symbols/bars) or saline (white symbols/bars) for 5 weeks. Analysis of total respiratory resistance responses measured at increasing doses of MCh in (A) WT HDM- and saline-exposed groups, (B) GATA HDM- and saline-exposed groups and (C) a composite of all 4 groups. Data show a time-course of 2 baseline measurements prior to nebulization of increasing doses of MCh followed by 12 consecutive measurements. $n = 6-7$ /group. Data are expressed as mean \pm SEM and are from one of three independent experiments that yielded similar results.

Figure E4. Impact of eosinophils on house dust mite (HDM)-specific serum IgE levels in PHIL mice. Separate groups of wild-type (WT) littermate control and PHIL eosinophil-deficient mice were exposed to HDM (black bars) or saline (white bars) for 5 weeks and sacrificed 72 hours after the last exposure. Graph shows HDM-specific Th2-associated IgE levels in the serum. $n = 3-7$ /group. Data are expressed as mean \pm SEM. $P < 0.05$ compared with the *corresponding saline-exposed group.

Figure E1

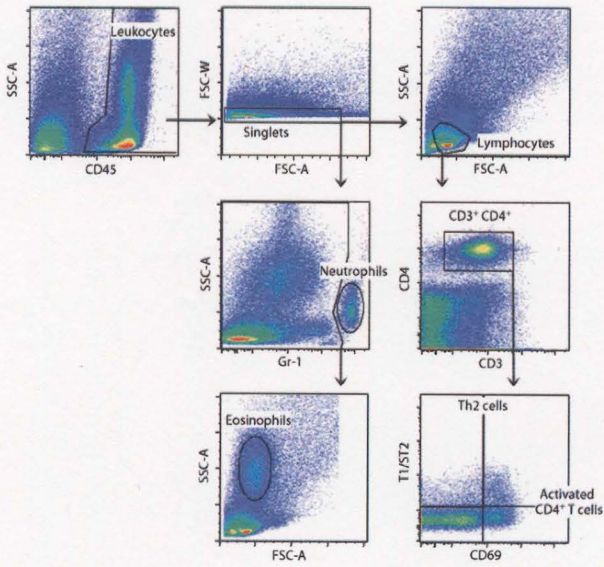


Figure E2

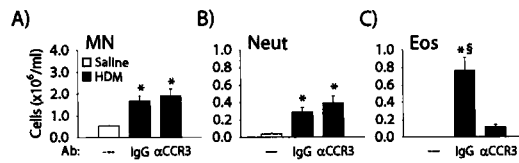


Figure E3

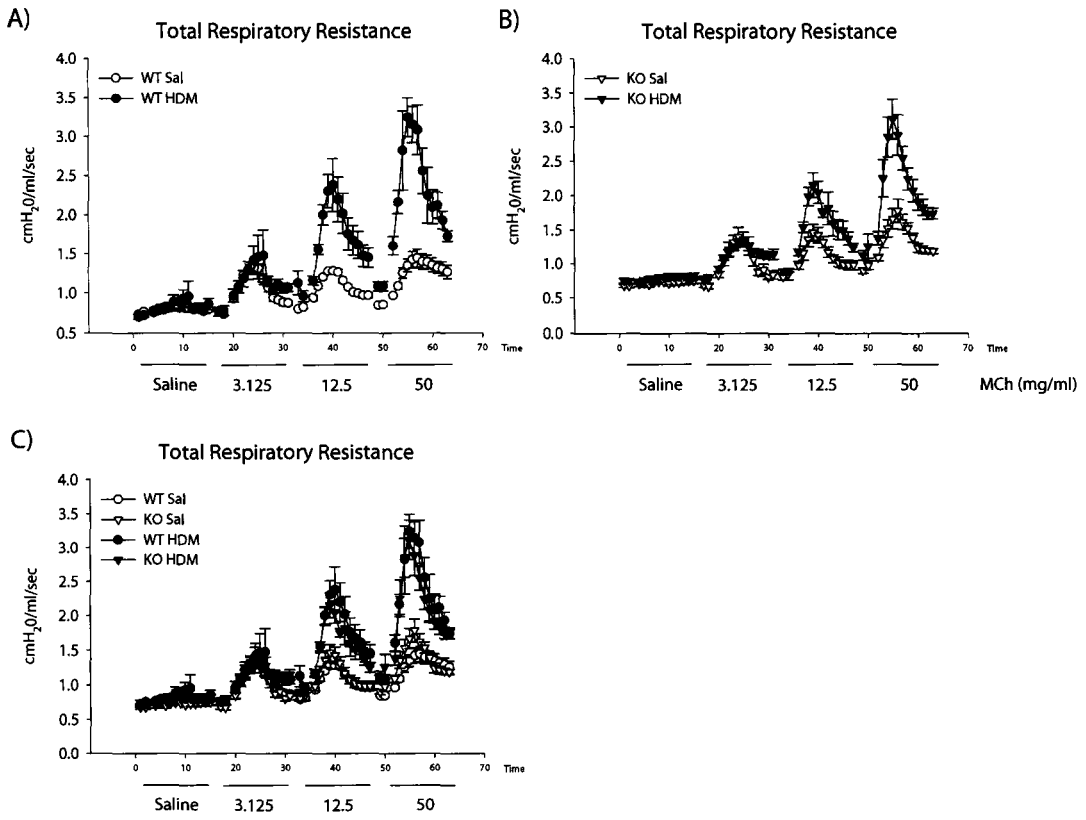
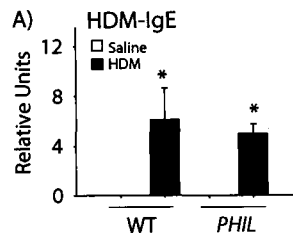


Figure E4



— Chapter 5 —

DISCUSSION

There is little question that allergic asthma is a chronic immune-inflammatory driven disease. Decades of *in vitro* and *in vivo* research have afforded us with considerable knowledge of the immunological mechanisms that underlie sensitization and govern the inflammatory response. Moreover, the immediate and short-term ramifications of the immune-inflammatory response on clinical symptoms and lung function have also been well defined. In contrast, the long-term consequences of continuous allergen exposure and persistent allergic immune-inflammatory responses on disease progression are, relatively speaking, less well understood. The reasons for this may stem, at least in part, from the difficulties associated with the study of chronic disease in humans, which takes many years to develop, and also, due to the availability of appropriate model systems, which only recently have been emerging. The work presented in Chapters 2–4 of this thesis have focused on studying the effects of continuous allergen exposure and persistent allergic immune-inflammatory responses on the development of adaptive Th2 responses to newly encountered antigens and the mechanisms by which persistent allergic inflammation may drive airway wall thickening. Together, these studies advance our understanding of the long-term impact of chronic allergen exposure and allergic inflammation on certain aspects of disease pathogenesis, namely, sensitization and airway remodeling.

Influencing Allergic Sensitization and Airway Inflammation

There are a number of factors (viruses, DEPs, tobacco smoke, etc...) that are able to promote the generation of an allergic immune-inflammatory response towards antigens intrinsically unable to induce such responses. The evidence indicates that these factors act by conditioning the lung microenvironment in a manner that favours the development of

type-2, instead of tolerogenic, adaptive immune responses. As aeroallergens are ubiquitously present in the environment and allergen exposure triggers an allergic immune response in sensitized individuals we questioned whether frequent exposure to a pervasive aeroallergen, such as HDM, may establish an airway milieu that biases immune responses to other inhaled Ags towards Th2. As was already discussed in Chapter 2, a limited number of previously published reports have investigated the potential of some allergens (e.g. Der p 1) to facilitate the development of allergic sensitization to OVA, the archetypical innocuous Ag (72, 175, 176). Collectively, the findings of those reports indicate that the immune response elicited by one allergen can promote sensitization (i.e. Ag-specific Th2-associated Igs) to other bystander antigens/allergens when encountered concomitantly.

In Chapter 2 we examined whether chronic HDM exposure could facilitate the full development of the ‘asthmatic phenotype’ towards OVA. In agreement with the previously published reports (72, 175, 176) we observed that mice concurrently exposed to a HDM extract with OVA for several weeks produced OVA-specific Th2-associated immunoglobulins and cytokines. More importantly, subsequent respiratory challenge with OVA alone elicited a robust allergic inflammatory response that was associated with a significant increase in AHR, demonstrating that HDM exposure subverted OVA’s innocuous nature and facilitated the development of overt allergic airway responses (177). In an extension of this work, concurrent HDM and OVA exposure was shown to potentiate some aspects of airway remodeling (178).

A major implication of these findings is that sustained immune-inflammatory responses to a given allergen may perpetuate allergic airway disease by broadening the spectrum of antigens to which an individual will react to. Certainly, allergic asthmatic individuals often display allergic reactivity towards a panoply of allergens and there is a tendency for this to build throughout life. In addition, there is evidence that atopic children born during times of unusually high spring pollen counts are more likely to become sensitized to a household pet than children born during a time when spring pollen counts were 'average' (179). These lines of evidence support the notion that sensitization to one allergen may facilitate sensitization to another in humans. However, given the myriad of potentially confounding factors, the extent to which allergen exposure and chronic immune-inflammatory responses may influence subsequent responses to newly encountered Ags/allergens may be difficult, if not impossible, to directly assess in humans. Moreover, our findings raise the question *why then are allergic asthmatics not reactive to all aeroallergens?* We suspect that the issue involves at least two factors: duration and timing of exposure. The data presented in Chapter 2 and our own unpublished data show that while five weeks of concurrent allergen exposure was sufficient to establish OVA-specific adaptive immune-inflammatory responses, a short-term coexposure protocol was not. Mice subjected to ten-days of HDM/OVA coexposure displayed only modest signs of OVA-specific Th2-polarized immunity and little, if any, inflammation following *in vivo* recall to OVA alone. Thus, protracted exposure periods are likely required for bystander antigen responses to fully develop. Timing may also be critical as the interval during which allergen exposure, and the subsequent allergic response, may bias the immune response to bystander antigens towards Th2 is likely to be limited. Therefore, although

humans are frequently exposed to multiple antigens, unless inhalation of a bystander antigen occurs within a given interval to allergen exposure, the default response (i.e. tolerance) is unlikely to be affected. Indeed, this has been shown to be the case, at least in experimental settings, with other factors that are able to facilitate sensitization and allergic airway responses, such as viruses (180, 181). The impact of timing was not directly examined in the work presented in Chapter 2 as the time between HDM and OVA exposures was held relatively constant in the exposure protocols employed. One way that the timing of exposure could be addressed would be to employ a recurrent HDM exposure model, such as that utilized in Chapter 3, where mice were sensitized and then subjected to several short (three-day) HDM rechallenges that were each separated by two-week rest periods. In this exposure system, the comparison of OVA-specific responses between i) mice subjected to OVA concurrently with HDM rechallenge and ii) mice subjected to OVA some time after (e.g. three days) HDM rechallenge, would allow for an assessment of the importance of timing.

TGF- β and Eosinophils in Allergic Airway Remodeling

The prevailing dogma is that TGF- β plays an integral role in the generation of allergic airway remodeling and that eosinophils are similarly important, on account of their ability to produce and/or activate TGF- β . This hypothesis was, until around 2004, based largely on three separate bodies of indirect evidence (discussed in Chapter 1). Since then, considerable research has been focused on directly examining the contribution of both TGF- β and eosinophils to the development of airway remodeling.

With respect to the role of TGF- β , experimental studies have clearly demonstrated that TGF- β is critically involved in the development of OVA-induced allergic airway remodeling. As it pertains to eosinophils, the bulk of the data suggest that eosinophils also play an integral role in the generation of the remodeled airway in OVA-systems, although this has not been without some conflicting results (discussed in Chapter 4). These findings have been taken as further evidence of the importance of TGF- β and eosinophils to remodeling and as a result, these two factors have become *principle targets* of future therapeutic strategies.

Admittedly, the evidence supports the *independent* involvement of both TGF- β and eosinophils in OVA-induced remodeling. However, there are a number of inconsistencies that, in fact, challenge the belief that, even in OVA systems, eosinophil-derived TGF- β is a predominant mechanism by which remodeling develops. Of the seven studies that examined the involvement of eosinophils in OVA-based systems, already elaborated upon in Chapter 1, five of them also investigated the cellular source of TGF- β in their systems. Using immunohistochemistry Cho *et al.* observed that while eosinophils comprised approximately sixty percent of TGF- β expressing peribronchial inflammatory cells, in chronically OVA challenged mice, macrophages made-up about thirty-five percent (115). Furthermore, they also noted “significant” TGF- β expression by the airway epithelium. Tanaka *et al.* conducted a kinetic analysis of TGF- β expression over their three-week OVA challenge protocol (120). Following one week of exposure they reported that among the lung inflammatory infiltrate mononuclear cells, alveolar macrophages and

“especially” eosinophils were producing TGF- β ; however, by the second and third weeks of exposure an increasing number of bronchial- and vascular-associated mesenchymal cells, particularly myofibroblasts, as well as alveolar epithelial cells became positive for TGF- β . The airway epithelium was reported to be the major site of TGF- β induction in a ‘low-level’ chronic OVA exposure model utilized in both the Kumar and Foster studies and, interestingly, they found no evidence of TGF- β expression by eosinophils (127). In addition, mononuclear cells, presumably macrophages, and not eosinophils were observed to be the main secretors of TGF- β during chronic OVA exposure in the Humbles study (182). Consistent with this, the authors also observed no differences in the expression of active TGF- β (mRNA or protein) between WT and Δ dblGATA eosinophil-deficient mice that were sensitized and chronically challenged to OVA (133). That the predominant cellular source of TGF- β appears to vary between these studies is intriguing. The findings of the Tanaka report suggest that the cellular source of TGF- β may change as the allergic response evolves from an acute process into a chronic/on-going one. It is also plausible that these differences can be attributed to variations in the exposure protocols used, e.g. high dose vs. low dose, three-week vs. twelve-week exposures, etc. Irrespectively, the above findings question the absolute importance of the eosinophil-TGF- β pathway in the development of airway remodeling. Thus, although TGF- β and eosinophils are integral mediators of OVA-induced remodeling, whether eosinophil-derived TGF- β is, universally, a major driving mechanism remains a contentious issue.

In Chapters 3 and 4 we show that neither TGF- β nor eosinophils are critically required for the generation of airway remodeling induced by continuous HDM exposure. Moreover, in the case of TGF- β , we further demonstrate that TGF- β blockade does not affect the development of airway remodeling in a model of intermittent HDM exposure; the difference being that this model induces remodeling in the setting of recurrent, transient, bouts of inflammation, and thus is, presumably, milder. The possible explanations that may account for the altered requirement of TGF- β and eosinophils between OVA and HDM systems have already been discussed in Chapters 3 and 4. Ultimately, it is clear that TGF- β and, perhaps to a lesser extent, eosinophils are required for the full development of remodeling in OVA models, however, it is equally clear that neither of these factors are necessary for remodeling to develop in a chronic HDM exposure system. What may be the significance of these findings? Arguably, they highlight the notion that the involvement of TGF- β and eosinophils in remodeling is contextual. Moreover, the findings in the HDM system force a re-evaluation of the concept that TGF- β and eosinophils are, unconditionally, essential mediators of allergic airway remodeling; with all the associated therapeutic implications.

One factor that was not examined in our experimental studies was the impact of the dose of HDM on the involvement of TGF- β and/or eosinophils in remodeling. The dose utilized throughout the studies presented here elicits a maximum, or near maximum, response in terms of lung inflammation and markers of systemic Th2 immunity (183). On the basis of our findings from the recurrent HDM model we surmise that, at least in the

case of TGF- β , remodeling would develop similarly (that is, independent of TGF- β) even if induced by lower doses of HDM, and hence, a milder inflammatory response. Still, the issue would be worthwhile to investigate as it is conceivable that the involvement of eosinophils and/or TGF- β is altered under different exposure conditions. That lower doses of HDM may change the requirement for these, and other, factors does not discredit the underlying central message of our findings, i.e. the notion of context-specific responses, but rather, further strengthens it.

Importantly, the contribution of eosinophils to allergic airway remodeling in humans is being investigated in clinical trials. In a limited but valuable study, the effect of short-term eosinophil depletion (accomplished via anti-IL-5 therapy) on ECM deposition was examined in a small group of mild atopic asthmatics (184). The authors reported that anti-IL-5 treatment, which partially depleted pulmonary eosinophilia, was associated with significant decreases in the expression of both tenascin and lumican compared to pre-treatment baselines and placebo controls. Moreover, they observed that the expression of procollagen III was also reduced relative to placebo. While these findings suggested a beneficial impact of eosinophil depletion on remodeling, there are a number of additional factors and study limitations that when taken into account strongly caution against any such conclusion from being made at this time. Indeed, it is important to note, as the authors did, that the observed decrease in procollagen III was statistically insignificant when compared to pre-treatment baseline measurements and that the pre-treatment baseline expression level of tenascin was considerably (ten fold) higher in the treated group versus placebo. Furthermore, the effects of eosinophil depletion on other key remodeling-

associated parameters, such as smooth muscle and overall basement membrane thickness, were not examined. Therefore, further investigation is likely required before the effects of eosinophil depletion on remodeling in humans can be clarified. Notably, no studies have reported on the impact of TGF- β blockade on airway remodeling in humans to date.

TGF- β and Eosinophils in Allergic Airway Inflammation and Immunity

The observation that both TGF- β and eosinophils are present in elevated amounts in the allergic lung is, at least in experimental systems, a consistent one. This, in light of our findings that neither TGF- β nor eosinophils are absolutely required for remodeling to develop in an HDM system, raises the question of what may be the contribution of these factors in the allergic response.

In the case of eosinophils, this question has been debated extensively and was considered in depth in Chapter 4. In the end, it seems that eosinophils are not fundamentally required for the development of allergic sensitization. Although some recent studies involving adoptive transfer of Ag-loaded eosinophils purport that eosinophils play an important role in the initial induction of Th2 immunity by acting as professional APCs (185, 186), as yet, the evidence obtained from loss-of-function studies (IL-5 blockade, eosinophil-deficient mice, etc...) demonstrates that in the absence of eosinophils sensitization is unaffected in models of allergic airway disease (133, 187, 188). In terms of the contribution of eosinophils to the inflammatory response, the findings indicate that eosinophils may augment leukocyte recruitment and mediator release.

However, the exact extent to which they may participate appears to vary depending on the experimental system and ranges from little, if any, to considerable.

With respect to TGF- β , our studies consistently demonstrated that impairing its activity resulted in exacerbated airway inflammation, due exclusively to an increase in eosinophilia, and heightened systemic Th2 responses, specifically HDM-specific Th2-affiliated Igs and splenocyte cytokine production (Chapter 3). These findings are in line with various *in vitro* studies that document the suppressive activity of TGF- β on macrophages (189, 190), lymphocytes (191-193) and eosinophils (194, 195), with effects ranging from inhibition of cytokine production and proliferation to apoptosis. In addition, our observations corroborate the findings of earlier reports in acute conventional OVA-based models that, collectively, demonstrated that impairment of TGF- β resulted in enhanced airway inflammation (mostly on account of eosinophils), OVA-specific immunity and, importantly, AHR (196-198).

In this regard, we also noted that TGF- β blockade was associated with augmented AHR. Initially, we tended to favour the explanation that the increase in AHR was a consequence of increased airway eosinophilia. However, the findings presented in Chapter 4, which show only a minor decrease in AHR in Δ dblGATA eosinophil-deficient mice subjected to chronic HDM exposure, suggest that this may not be the case. Instead, it is perhaps more likely that TGF- β impairment potentiated some component of the immune-inflammatory response, perhaps cytokine production by T cells and/or macrophages which, in turn, amplified AHR. Although we were unable to provide

evidence of an enhanced cytokine response in the lung, on account of some technical limitations, there is evidence to support this notion. We documented increases in IL-5 and IL-13 production by splenocytes harvested from HDM-exposed anti-TGF- β treated mice upon re-stimulation with HDM *in vitro*, relative to positive controls. Moreover, Alcorn *et al.* reported that anti-TGF- β 1 treatment significantly increased the levels of Th2 cytokines in the BAL of OVA exposed mice and enhanced AHR in the absence of any effect on airway inflammation (160).

Interestingly, in the McMillan study, TGF- β blockade during the chronic exposure phase of their OVA-based model had absolutely no effect on the allergic inflammatory response (118). While there is no reason to doubt this finding, we suggest that it does not necessarily absolve TGF- β of a role as a negative regulator of allergic inflammation. Instead, we suspect that the reported lack of effect may be due to the dose of anti-TGF- β Ab used in their study. In fact, we repeated their experiment, but treated mice with a higher dose of anti-TGF- β . Under these conditions, we observed a significant increase in airway inflammation and eosinophilia (unpublished results). Thus, we contend that even in this experimental system TGF- β is indeed a negative regulator of the allergic inflammatory response.

Potential Role of IL-13

The finding that airway remodeling can develop independently of TGF- β in the context of HDM exposure compels us to contemplate the question of what factor(s), other than TGF- β , may drive the development of HDM-induced remodeling. As was

mentioned previously, a number of molecules have been implicated in this process, of which IL-13 may be particularly relevant on account of its involvement in Th2 immunity.

Experimental studies in OVA models have consistently demonstrated that IL-13-deficient mice and WT mice treated throughout challenge with an IL-13 neutralizing reagent are, for the most part, protected from the generation of airway remodeling (80, 199, 200). Similarly, IL-13 inhibition was shown to abrogate increases in mucus production and subepithelial collagen deposition in an *Aspergillus fumigatus* model of allergic airway disease that was induced by conventional means (201). Moreover, constitutive pulmonary expression of IL-13 alone was enough to trigger certain aspects of remodeling including mucus hypersecretion and subepithelial fibrosis (122). While these findings strongly support the idea that IL-13 contributes to the development of airway remodeling, it has been suggested that IL-13 may influence remodeling-associated processes such as ECM deposition and smooth muscle thickening only indirectly, via the induction of TGF- β (127, 202). However, it should be noted that IL-13 can, in fact, elicit various remodeling-associated responses independently of TGF- β . *In vitro* studies have shown that IL-13 can stimulate myofibroblast differentiation and proliferation, activate human airway fibroblasts and induce collagen production (203–207). Furthermore, IL-13 injection into the peritoneal cavity of two-week old TGF- β 1-deficient mice triggered the expression of various genes associated with the repair/remodeling response including collagen I and III, tenascin, TIMP-1 and MMP's to levels that were equivalent as those in IL-13-injected WT mice (208). Of particular interest, *Schistosoma*-induced hepatic fibrosis, which develops as a consequence of a robust Th2-polarized inflammatory

response, was found to occur in an IL-13-dependent, TGF- β -independent manner (208). Thus, on the basis of these findings, it is plausible that an IL-13-dependent (TGF- β -independent) mechanism may be mediating the HDM-induced remodeling response. Ultimately, further investigations in models employing CEA's, such as HDM, are required to determine whether IL-13, unlike TGF- β (and eosinophils), mediates allergic airway remodeling in non-OVA-based models.

We have considered the possibility that HDM-induced remodeling may not be driven by any one single factor, but rather, by the redundant activities of a few different factors. Certainly, it is by no means a coincidence that the current 'Gold Standard' treatment in asthma, corticosteroids, achieves their beneficial effects by influencing multiple immune-inflammatory pathways. As yet, we have not investigated this issue experimentally, although, conceivably such experiments could be approached by various strategies. For example, the possibility that IL-13 and TGF- β may play redundant roles in HDM-induced remodeling could be examined by treating Smad3-deficient mice with an IL-13 neutralizing reagent, or, by treating WT mice with a combination of both IL-13 and TGF- β neutralizing reagents. The prospect of redundancy is consistent with not only the data we present in Chapters 3 and 4 but also with a basic operating principle of biological responses: a degree of redundancy to ensure survival. Most importantly, this notion carries considerable implications with respect to the design of novel therapeutic strategies for the treatment of allergic asthma.

Summary and Concluding Thoughts

In summary, the research documented in Chapters 2-4 of this thesis have centered on investigation of the impact of chronic allergen exposure and allergic airway inflammation on some aspects of the pathogenesis of allergic asthma. The underlying goal was that this research would inform future therapeutic strategies. In this regard, the work presented here conveys several practical and conceptual messages.

Studies in a model of concurrent allergen exposure show that some allergenic materials, such as HDM, can condition the airway environment in such a way that fosters sensitization and overt allergic responses to other antigens/allergens. Thus, chronic aeroallergen exposure, like many other biological and environmental factors, may perpetuate allergic airway disease by encouraging poly-sensitization.

Evidently, the data pertaining to TGF- β 's involvement in the development of remodeling, induced by chronic OVA and HDM exposure, are divergent. On the other hand, investigations in both short- and long-term exposure models, employing HDM or OVA, consistently show that TGF- β acts as a negative regulator of allergic inflammation. This, we suggest may be the central role of TGF- β in allergic airway disease. These findings, from the perspective of therapy, indicate that the benefit of impairing TGF- β function on remodeling is unclear, at best, and moreover, that any such intervention strategy may in fact aggravate airway inflammation and worsen lung function.

The therapeutic benefit of eosinophil depletion on remodeling also seems uncertain based on the experimental data from both OVA and HDM studies and the

limited clinical trial data. However, the data suggest that eosinophils augment airway inflammation and thus therapeutic ablation strategies may provide some clinical benefit. Importantly, the clinical impact of eosinophils in allergic asthma is now being investigated in human trials. The most recent evidence indicates that eosinophil depletion reduces the number of asthma exacerbations, but, does little to improve symptoms or AHR, at least in certain selected asthma populations (209, 210).

The findings of the TGF- β and eosinophil HDM-studies also bring to fore two central concepts: the notion of context specific responses and the importance of embracing heterogeneity. Already mentioned in brief is the idea of context. In essence the message is that just because one can achieve the same phenotypes (allergic inflammation, remodeling, AHR), using different systems (HDM, OVA/Alum, etc.), does not imply the same underlying mechanisms. The inherent connotation is that there may be multiple pathways by which allergic responses can be initiated. In fact, evidence in support of this notion is now emerging. In this regard, investigations have shown that the aluminium-based adjuvants employed in conventional OVA models trigger allergic responses through an IL-1/uric-acid/NALP3-inflammasome mediated process (211-214), whereas Der p 2, elicits Th2-associated responses via a mechanism involving molecular mimicry of the TLR-4-associated molecule MD-2 (77). Moreover, papain, a proteolytic enzyme, establishes Th2-immunity via the actions of basophils and possibly TSLP (thymic stromal lymphopoietin) (215, 216) and chitin-induced Th2-polarized inflammation was found to be mediated, in part, by alternative macrophage activation and the production of leukotriene B₄ (217). Therefore, it follows that the network of responses elicited by different aeroallergen

exposures, i.e. ragweed, roaches, fungi or otherwise, is likely to vary; perhaps, according to the properties of its constituents be they limited or diverse in composition, rich or poor in TLR-stimulating or proteolytic agents, etc. This rationale suggests that no one experimental model can represent the range of responses elicited by allergen exposure, especially when taking into account that ‘real-life’ exposure likely occurs in combination with various additional biological and non-biological entities. Thus, the heterogeneity of responses elicited by different model systems must be embraced, as it is likely a reflection of the heterogeneity of human asthma. Arguably, this line of thought insinuates that there are unlikely to be any simple answers about the role of a particular cell type or molecule in asthma pathogenesis, or, the effect of a specific therapy on clinical outcome. Importantly, this predisposition informs that a more productive endeavour would be to uncover the full spectrum of asthma heterogeneity, in both the real world and experimental systems, as a way of decoding the conditions under which a particular molecule, cell or therapy may have a distinctive impact.

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