MODELING AND MECHANISTIC INSIGHTS INTO THE DEVELOPMENT OF ALLERGIC AIRWAY RESPONSES TO HOUSE DUST MITE

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MODELING AND MECHANISTIC INSIGHTS INTO THE DEVELOPMENT OF ALLERGIC AIRWAY RESPONSES TO HOUSE DUST MITE

By

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Abstract

Allergic asthma is a chronic and complex disease of the airways characterized by dysregulated immune-inflammatory responses to aeroallergens and reversible airflow obstruction. The prevalence and economic burden of allergic asthma have increased substantially over the last five decades. Despite remarkable progress in our understanding of the immunobiology and pathophysiology of asthma, the ontogeny of the disease remains elusive. As a result, there is a lack of effective preventative strategies. Here, we used a murine model of allergic asthma to house dust mite (HDM), the most pervasive indoor aeroallergen worldwide to address issues pertaining to the development of allergic asthma. First, we provided a comprehensive computational view of the impact of dose and length of HDM exposure on both local and systemic allergic outcomes (Chapter 2). Parameters, such as thresholds of responsiveness, and non-linear relationships between allergen exposure, allergic sensitization and airway inflammation were identified. We, then, investigated molecular signatures implicated in the onset of allergic responses (Chapter 3). HDM exposure was associated with production of the epithelial-associated cytokines TSLP, IL-25 and IL-33. However, only IL-33 signaling was necessary for intact Th2 immunity to HDM, likely because of its superior ability to induce the critical co-stimulatory molecule OX40L on dendritic cells and expand innate lymphoid cells. Lastly, as individuals are most likely exposed to allergens concomitantly to other environmental immunogenic agents, we studied the impact of an initial immune perturbation on allergic responses to sub-threshold amounts of HDM (Chapter 4). We showed that transient expression of GM-CSF in the airway substantially lowers the threshold of allergen required to generate robust,

HDM-specific Th2 immunity, likely through increasing IL-33 production from alveolar type II cells. These studies favor a paradigm whereby distinct molecular pathways can elicit type 2 immunity, intimating the need to classify asthma into distinct clinical subsets.

Acknowledgements

I have experienced a fabulous journey over the past seven years. Studying for my PhD in Canada has been a great challenge but I have gained the most in my life. I truly believe that such an achievement could not have been accomplished alone and, thus, everyone mentioned here deserves to share my delight. I have many people to show my great appreciation.

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I am especially grateful to my committee members, Drs. Martin Stämpfli, Anthony J. Coyle and Paul M. O'Byrne, for their helpful discussions and suggestions. I deeply appreciate Martin for guiding me professionally and personally, and for counting on me for some of the research performed in his lab. I would like to express appreciation to Dr. Alison Humbles and her team at MedImmune LLC (Gaithersburg, MD, USA) for generously sharing data and her deep knowledge of the field, providing reagents and mice… and organizing the ATS dinners! To Dr. Lennart K. Lundblad at the University of Vermont for his generous assistance and helpful answers to my never ending questions about lung physiology and the *flexi*Vent system.

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are truly inspirational. Thanks to Susanna and Tina, who have taught and helped me with many of the *in vitro* and *in vivo* techniques that were required for this project… and for their unparalleled support when moving in and out of Canada. I am also grateful to all undergraduate and visiting students who spent time in our lab and contributed to my research. To Mary Kiriakopoulos, Marie Bailey and the secretarial staff for all their assistance. Another thank you to all of the staff at the McMaster's Central Animal Facility as well as neighboring laboratories for help and productive collaborations, namely the Stämpfli, Richards, Xing, Bowdish and Ashkar groups, at the McMaster Immunology Research Centre.

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List of Abbreviations

The following abbreviations were used in addition to those abbreviations commonly accepted for units of measure and quantities.

Ab antibody
AHR airway hyperresponsiveness
APC antigen-presenting cell
ATII type 2 alveolar epithelial cell
BAL bronchoalveolar lavage
BMDC bone marrow-derived DC
BSA bovine serum albumin
CD cluster of differentiation
d day
DC dendritic cell
EC epithelial cell
ELISA enzyme-linked immunosorbent assay
FBS fetal bovine serum
GM-CSF granulocyte-macrophage colony-stimulating factor
HDM house dust mite
IFN interferon
Ig immunoglobulin
IL interleukin
IL-17RB IL-17 receptor B
ILC type 2 innate lymphoid cell
i.n. intranasal
i.p. intraperitoneal
i.n. intratracheal
i.v. intravenous
KO knockout
LN lymph node
LPS lipopolysaccharide
mDC myeloid DC
MHC major histocompatibility complex
NKT natural killer T cell
OVA ovalbumin
OX40L OX40 ligand
PN peanut
r recombinant
STAT signal transducer and activator of transcription
Th T helper cell (Th2 for type 2 Th)
TLR toll-like receptor
TNF tumor necrosis factor
TSLP thymic stromal lymphopoietin
TSLPR TSLP receptor
wk week
WHO World Health Organization
WT wild-type

Declaration of Academic Achievement

In accordance with the *Guide for the Preparation of Theses at McMaster University*, the research documented herein is presented as a "sandwich doctoral thesis". The articles presented in Chapters 2, 3 and 4 are three independent although conceptually related bodies of work that, as of February 2013, have been or are being peer-reviewed prior to publication. The work conducted in each manuscript required a collaborative effort with several colleagues, resulting in multiple authors. As such, my contributions to each article are highlighted below.

Chapter 2. **Llop-Guevara A***, Colangelo M*, Chu DK, Moore CL, Stieber NA, Walker TD, Goncharova S, Coyle AJ, Lundblad LK, O'Byrne PM, Lovric M, Jordana M. *In Vivo*-to-*In Silico* Iterations to Investigate Aeroallergen-Host Interactions. ***PLoS One***. **2008** Jun 11;3(6):e2426. doi: 10.1371/journal.pone.0002426.

This work was conducted over the period of January 2006 – December 2007. This manuscript was an equal contribution (*). I designed and performed all the *in vivo* experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. M. Colangelo developed the mathematical equations, performed the *in silico* simulations and also participated in the preparation and writing of the manuscript. Experimental assistance was provided by undergradute students that I supervised (D.K. Chu, C.L. Moore and N.A. Stieber) and technicians (T.D. Walker and S. Goncharova). Drs. L.K.A. Lundblad and M. Lovric provided valuable scientific input in lung physiology and mathematical modeling, respectively. Drs. A.J. Coyle and P. O'Byrne critically appraised the manuscript. Dr. Jordana, along with A.L-G and M.C., conceived and designed the experiments and also revised the paper and the responses to comments from the reviewers.

Chapter 3. Chu DK*, **Llop-Guevara A***, Walker TD, Flader K, Goncharova S, Boudreau JE, Moore CL, In TS, Waserman S, Coyle AJ, Kolbeck R, Humbles AA, Jordana M. IL-33, but not Thymic Stromal Lymphopoietin or IL-25, is Central to Mite and Peanut Allergic Sensitization. ***J Allergy***

Clin Immunol. **2013** Jan;131(1):187-200.e8. doi: 10.1016/j.jaci.2012.08.002. Epub 2012 Sep 21.

This work was conducted over the period of March 2009 – December 2011. This manuscript was an equal contribution (*). I, along with D.K. Chu (graduate student), designed and performed all experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. I led the work related to allergic responses to house dust mite, while D.K. Chu guided the work in peanut allergy and anaphylaxis. Experimental assistance was provided by undergradute students that I supervised (C.L. Moore and T.S. In), graduate students (K. Flader and J.E. Boudreau) and technicians (T.D. Walker and S. Goncharova). Drs. R. Kolbeck and A.A. Humbles (MedImmune collaborators) kindly provided reagents, knock-out mice and scientific input. Drs. S. Waserman and A.J. Coyle critically appraised the manuscript. Dr. Jordana, along with D.K. Chu and myself, conceived and designed the experiments and also revised the paper and the responses to comments from the reviewers.

Chapter 4. **Llop-Guevara A**, Chu DK, Walker TD, Goncharova S, Fattouh R, Silver JS, Moore CL, Xie JL, O'Byrne PM, Coyle AJ, Kolbeck R, Humbles AA, Stämpfli MR, Jordana M. A GM-CSF/IL-33 Pathway Facilitates Allergic Airway Responses to Sub-Threshold House Dust Mite Exposure. Submitted in March 2013 to *Mucosal Immunol*.

This work was conducted over the period of October 2007 – February 2013. I designed and performed all experiments (except the ones in Figure 7a,b), analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. Experimental assistance was provided by graduate students (D.K. Chu and R. Fattouh), undergradute students that I supervised (C.L. Moore and J.L. Xie) and technicians (T.D. Walker and S. Goncharova). Drs. R. Kolbeck, J. Silver and A.A. Humbles (MedImmune collaborators) kindly provided knock-out mice and performed the *in vitro* assays with epithelial cells (Figure 7a,b). Drs. P.M. O'Byrne, A.J. Coyle and

M.R. Stämpfli provided valuable scientific input. Dr. Jordana, along with myself, conceived and designed the experiments and revised the paper.

In addition to these three manuscripts, some of the work/concepts presented in this thesis were contributed to two book chapters. These are included as Appendices I and II.

Appendix I. Cates EC, Fattouh R, Johnson JR, **Llop-Guevara A**, Jordana M. Modeling Responses to Respiratory House Dust Mite Exposure. **Contrib Microbiol. 2007**. Volume 14 (42-67): *Models of Exacerbations in Asthma and COPD.* Eds. Sjobring U, Taylor JD. Basel, Karger. doi:10.1159/000107054

This paper was researched and written over the period of June 2006 – December 2006. My contributions to this work included evaluating the published literature, and structuring and writing the article in collaboration with all other authors.

Appendix II. **Llop-Guevara A**, Marcinko J, Fattouh R, Jordana M. Interleukin 3, Interleukin 5, and Granulocyte–Macrophage Colony-Stimulating Factor. **2011**. Chapter 14 in *Inflammation and Allergy Drug Design*. Eds Izuhara K, Holgate ST and Wills-Karp M. Wiley-Blackwell. doi: 10.1002/9781444346688.ch14

This paper was researched and written over the period of June 2009 – January 2010. As the leader of this work, my contributions included evaluating the published literature and structuring and writing the article in collaboration with all other authors.

Chapter 1. Introduction

Overview of allergic asthma

Allergic asthma in numbers

Asthma is one of the most common chronic diseases, affecting around 300 million people of all ages worldwide (1,2). Asthma is closely linked to allergies; about 50 % of asthmatics older than 30 years of age are allergic, and younger asthmatics have an even higher incidence of allergies (up to 90 %). The prevalence of asthma has increased over the last 50 years; it currently varies widely (1 to 18 % of the population) among different countries (1). For example, the prevalence in Denmark and Greece is below 5 %, is approximately 10 % in Ontario (Canada), and it is over 15 % in Ireland and New Zealand (1,3,4). The rise in asthma worldwide has been observed in both children and adults, and is positively associated with the adoption of modern lifestyles and urbanization (5). With the emergence of new developed countries, the World Health Organization (WHO) estimates that the overall prevalence of asthma will continue increasing to over 400 million by 2025 (1). While the prevalence of asthmatics is rising in low and middle income countries, it is plateauing, and even decreasing, in high income countries, with a high prevalence of the disease (6,7) (Figure 1).

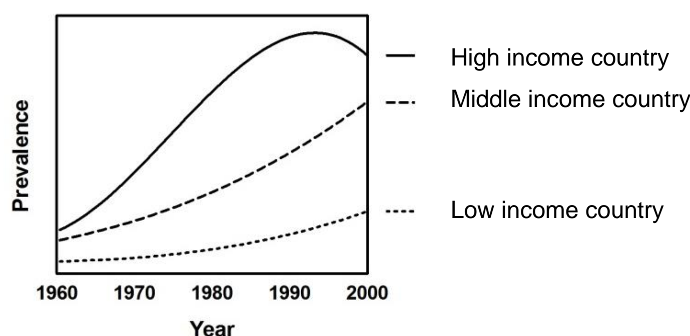

Figure 1. *Asthma prevalence trends by type of country. Adapted from WHO (1).*

Asthma significantly impacts quality of life. Among others, asthma affects daily activities, results in absences from school/work and increases hospital visits and patient admissions. The economic costs associated with asthma are among the highest

for non-communicable diseases because of the substantial health service use, in many cases over a lifetime, and loss of productivity. In the United States and Canada, for example, annual care costs exceed $18 billion and $600 million, respectively (8,9). Moreover, the WHO estimates that over 250,000 people die prematurely each year from asthma. Many of the deaths are preventable as a result of suboptimal long-term medical care and delay in obtaining help during severe attacks. Therefore, the highest death rates occur in those countries where health care and treatment are less available (1,2).

Allergic asthma in the clinic

The term asthma comes from the Greek verb *aázein*, meaning "to pant, to exhale with the open mouth, to breath hard". It seems that the word *ásthma* was first used as a medical term by Hippocrates *circa* 450 BC (10). It was not until early in the 20th century that the term *allergie*, which stems from the Greek *állos* meaning "other, changed or altered state" and *érgon* meaning "activity, reaction or reactivity", was introduced (11). Asthma is currently understood as a chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm (12). Allergic asthma, also known as atopic or extrinsic asthma, is the most common type of asthma and develops from a hypersensitivity reaction of the immune system to inhaled antigens usually present in pollens, dusts, animal dander or molds. Asthmatic symptoms include sudden attacks of labored breathing (dyspnea), chest constriction, wheezing, coughing and airway hyperresponsiveness (AHR) to environmental stimuli (12,13).

AHR is a term that encompasses changes in airway sensitivity, reactivity and maximum airway narrowing, all of which are often proportionate to the severity of the disease (13-16). Increased airway sensitivity refers to the tendency of the airways to contract to concentrations of bronchoconstrictors, such as histamine or methacholine, which would not cause airway narrowing in normal individuals. Increased airway reactivity is an index of the degree of change in airway tone for a given increase in the concentration of the agonist. An individual asthmatic will also show increased maximum airway narrowing as compared to non-asthmatics.

Persistent (structural changes in the airways) and variable (inflammation) features have been associated with AHR (12,13,16). Inflammatory events in the airway are temporary and influenced by numerous environmental stimuli. The inflammatory cell types commonly associated with the asthma phenotype include eosinophils, mast cells and $CD4^+$ T cells, in particular type 2 T helper cells (Th2 cells). However, there is heterogeneity among asthmatics as evidenced by varying inflammatory profiles, such as eosinophilic, neutrophilic, mixed (with both eosinophils and neutrophils) or pauci-granulocytic (with few or no granulocytes in the sputum) (17). Inflammation is thought to play a role in initiating and perpetuating airway remodeling, a term that describes structural changes to multiple components of the airway wall, such as sub-endothelial and sub-basement membrane thickening, loss of epithelial integrity, goblet cell meta/hyperplasia with mucus production, smooth muscle meta/hyperplasia, matrix deposition and angiogenesis (12,16,18). These changes in the architecture of the airways make them thicker, less compliant and narrower. Therefore, inflammation, remodeling and AHR are likely interdependent processes. Dissecting the relative contribution of structural and inflammatory changes to each component of AHR will provide greater insights into the mechanisms of asthma and the use of specific therapeutic strategies.

Available therapeutic strategies are unable to prevent the development of, or cure, asthma, but are effective for the management of the disease in most patients (19,20). For example, inhaled short-acting β2 agonists, such as salbutamol, are used to alleviate acute symptoms, and corticosteroids, long-acting β2 agonists, mast cell stabilizers or leukotriene antagonists, are prescribed for long-term control. In patients with persistent asthma, identification of precipitating allergens and subsequent reduction in exposure can also improve symptoms and reduce the requirement for medications (20-23). Avoidance measures for dust mite allergy include moving residence to low allergen environments (high altitude, hospital rooms), cleaning (vacuum with HEPA filters and wash bedding in hot water), removing dust reservoirs (carpets, stuffed animals) and decreasing humidity and temperature at home. Evidence regarding the potential benefit of allergen avoidance in preventing asthma is controversial. Some studies have shown a correlation between the level of house dust

mite (HDM) exposure early in life and the extent of sensitization and asthma symptoms (24). Others, though, have reported no effect (25), increased immunoglobulin (Ig) E sensitization (26) or even suggested a delay rather than prevention of asthma development (27). A potential explanation is that extremely low allergen exposures can lead to sensitization in susceptible individuals and, as a result, anything less than complete allergen avoidance is unlikely to be successful. Finally, specific immunotherapy, which aims at increasing immunological tolerance and the induction of blocking IgG4 through repeated exposure to allergen(s) (19), has been shown to benefit selected groups of asthmatics allergic to HDM, cat and grass pollen (28). In addition, immunotherapy has also been found to decrease the development of sensitization to new allergens and the incidence of new asthma cases in children with allergic rhinitis, which often precedes asthma (19,29). Further clinical trials will need to confirm the efficacy and safety of immunotherapy. Additional strategies are being and will need to be investigated to help improve asthma control and reduce the risk of exacerbations in patients with uncontrolled asthma (30). Clearly, there is a need for a better understanding of the origins of allergic asthma and the factors responsible for its increasing incidence with a view to developing effective primary preventative measures.

Immunobiology of allergic asthma

From an immunological perspective, the development of allergic asthma can be separated into two distinct stages: an *induction or sensitization phase*, which primes the immune system for an allergic response, and an *elicitation phase*, in which this response is triggered (Fig. 2).

Sensitization refers to the initial immunological process whereby an individual acquires the ability to mount an Ag-specific immune response, *i.e.* activates adaptive immunity and acquires immunological memory to a given antigen. The classical initiation of an allergic phenotype requires that professional antigen-presenting cells (APC), of which dendritic cells (DCs) are the most powerful, internalize and process foreign antigens resulting in the presentation of peptides via MHC class II (MHC II) molecules on the cell surface (31). A foreign antigen is described as an allergen once

an adaptive immune response is developed against the given molecule. Examples of common allergens are the HDM protein Der p 1, and the cat protein Fel d 1.

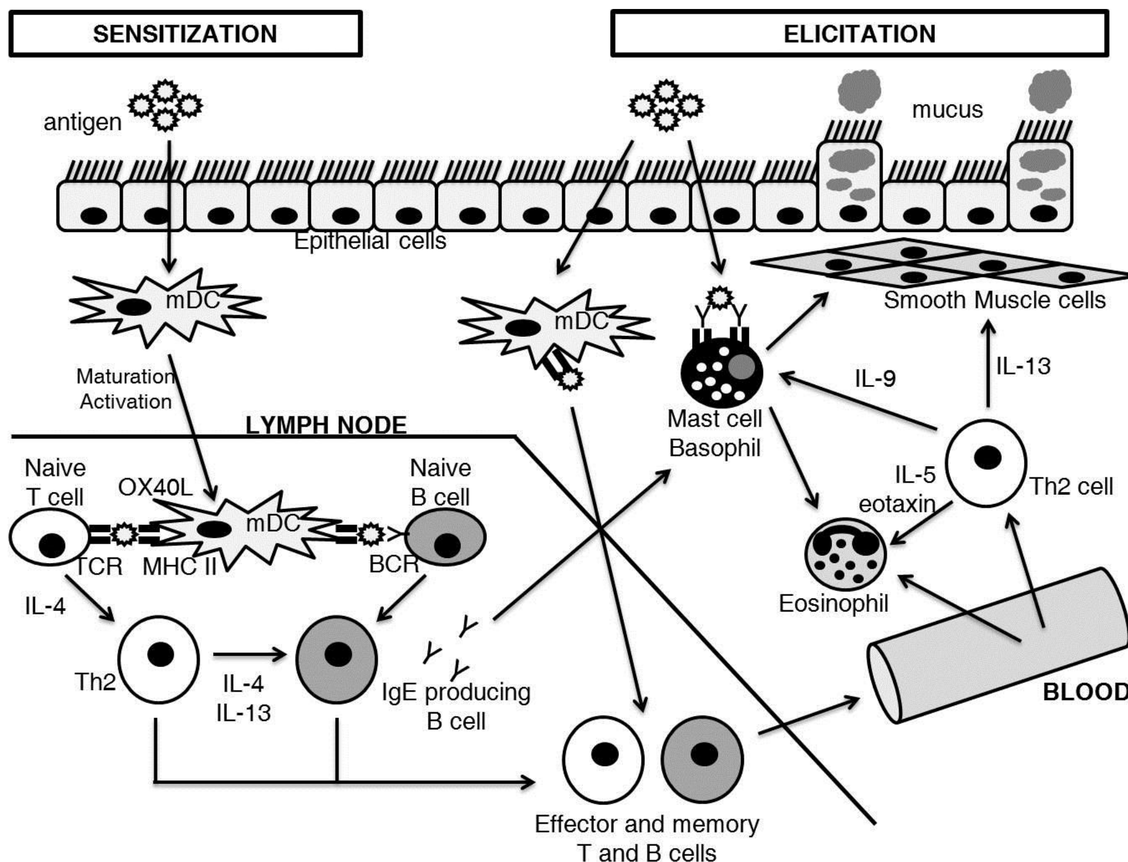

Figure 2. *Schematic representing a summary of events underlying allergic asthma pathogenesis. A) Initial exposure and sensitization to an allergen. B) Elicitation of responses upon allergen re-exposure resulting in lung eosinophilic inflammation and airway remodeling with mucus hypersecretion and smooth muscle cell hyperreactivity.*

Activated DCs then migrate to draining lymph nodes, where they present foreign peptides in the context of MHC II along with co-stimulatory signals to naïve $CD4^+$ T cells and B cells with their varied receptors (TCRs and BCRs). The differentiation of naïve T cells into functional T cell subsets (*e.g.* T helper type 1 (Th1), Th2, Th17, regulatory T cells) depends largely on the type of antigen being presented and the signals (cytokines and co-stimulatory molecules) present in the microenvironment. In

allergy, there is typically development of Th2 cells, which requires interleukin 4 (IL-4) and the subsequent activation of the transcription factors STAT6 and GATA-3 (32-34). Indeed, we showed that IL-4-deficient mice elicit not a Th2 but a Th1-polarized immune response to HDM that is not associated with typical asthma features (35). While IL-4 is critically required for the development of IL-4-expressing Th2 cells, the initial cell source of IL-4 remains to be clarified (reviewed in (36-38)). Several innate cells, including γδ T cells, eosinophils, basophils, natural killer T (NKT) cells and mast cells, as well as naïve $CD4^+$ T cells have been shown to be capable of rapid IL-4 production. In HDM allergy, eosinophils (39) and basophils (40) are not required for allergic sensitization, and both mast cells (41) and NKT cells (42) are thought to mediate the effector response rather than the induction of Th2 cell differentiation. Similarly, recent investigations have shown that the novel type 2 innate lymphoid cells (ILC), stimulated by epithelial cell (EC)-derived cytokines (primarily IL-25 and IL-33), can promptly produce IL-5, IL-13 and, to a lesser extent, IL-4 (43-46). However, Liang *et al.* have suggested that these cells likely function to orchestrate the effector, inflammatory response (47). Upon Th2 polarization, effector Th2 cells proliferate in the lymph nodes and migrate to the initial site of exposure where they produce Th2 cytokines and chemokines, such as IL-4, IL-5, IL-13 and eotaxin. Within the lymph node follicles, a specific subset of activated $CD4^+$ T cells provides key help (via IL-4/IL-13 and co-stimulation) to activated B cells to proliferate and undergo somatic hypermutation and Ig isotype class switching to antigen-specific IgG1, IgA and IgE (48,49). Circulating antigen-specific IgE will bind to cells in tissues expressing the high-affinity receptor FcεRI, including mast cells and basophils, or the low-affinity receptor FcεRII (*a.k.a.* CD23), such as B cells, eosinophils and macrophages. DCs express both FcεR I and II, which have been shown to enhance allergen uptake and the ensuing allergic response (50). Finally, there is formation of a pool of allergen-specific memory $CD4^+$ T cells and B cells, which will require low stimulation to become activated. Importantly, the initiation and successful execution of these events during the sensitization phase is thought to largely depend on the conditioning imposed by the lung microenvironment on APCs at the time of initial allergen encounter. This

thesis aims, in part, at investigating the molecular signals and mechanisms responsible for driving allergic sensitization.

Subsequent allergen exposure in sensitized individuals leads to cross-linking of FcεR I receptors by antigen binding to IgE resulting in mast cell and basophil activation and release of various granule-associated and/or newly generated mediators (49). Early mediators, such as histamine, prostaglandins, tryptase and leukotrienes, elicit transient changes in the airway, including airway smooth muscle contraction, mucus secretion, dilation of blood vessels, increased vascular permeability and expression of adhesion molecules on endothelial cells. As a result, there is airway narrowing as well as activation and recruitment of inflammatory leukocytes. Cross-linking of FcεR II results in the amplification of the immune response (51). This is known as the early asthmatic response, which occurs within minutes and is usually followed by a restoration of normal respiratory function within 1-3 h. However, the immune system simultaneously mounts a complex and prolonged response that can cause a relapse in pulmonary function 6-12 h after exposure and can last for over 24 h, known as late asthmatic response (19,52). Resident DCs capture allergen and activate memory Th2 cells and B cells to release inflammatory mediators. As reviewed in Appendix II, the Th2 cytokines IL-3 and IL-5 as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulate eosinophil production and mobilization from the bone marrow and their activation and survival in the lung (53). Lung infiltration of eosinophils is one of the hallmark features of allergic disease (19). Eosinophils are thought to contribute to tissue damage by the release of major basic protein, eosinophil cationic protein and leukotrienes, among other mediators. Monocytes, neutrophils and basophils are also recruited and activated for mediator secretion, further contributing to the inflammatory response. As most aeroallergens are ubiquitous in nature, allergen re-exposures can lead to chronic airway inflammation, which is postulated to influence airway remodeling and culminate in long-lasting impaired airflow and the clinical symptoms of allergic asthma mentioned above (19,52).

Modeling allergic asthma

Modeling in perspective

The acquisition of knowledge in biology has grown exponentially thanks in part to technological advances, the convergence of scientific disciplines and the use of modeling approaches. Models are physical, conceptual or computer-based representations of real-world entities. They are used across disciplines as they are simpler to understand and manipulate. Models are particularly useful to make observations, understand causality and project future events. Indeed, modeling is an indispensable part of the scientific enterprise.

Biological systems are considered to be complex, as they include many elements that can dynamically interact. In this case, *in vivo*, *in vitro* and, more recently, *in silico* models help to understand a complex biological system as a whole and discover emergent properties (54,55). *In vivo* animal modeling usually refers to the use of whole, living organisms other than humans as surrogates for studying human pathologies and health. When *in vivo* models are still too complex to identify individual components and their basic biological functions, *in vitro* systems are used, where isolated parts of an organism are maintained in controlled environments. In contrast, *in silico* models merge mathematical equations that describe the behavior of a biological system with computer simulations that allow making predictions and computer-based experiments. *In silico* models have the potential to speed up the rate of discovery while reducing the need for expensive lab work and clinical trials.

Complex systems are more than the sum of their parts and, consequently, any model of the structure of a complex system is necessarily incomplete. Therefore, it becomes critical to determine the factors that are important in the system and must be included in the model. Then, model users need to understand the original purpose and the assumptions made that are pertinent to its validity. Indeed, models must be consistent with empirical data; any model inconsistent with reproducible observations must be modified or rejected. Valid models allow for generating and testing hypotheses and predictions (56). In addition, as exemplified in Chapter 2 of this thesis, *in silico* models allow for the visualization of extensive arrays of previously

unknown responses and behaviors in a way, such as in 2 and 3-dimensions, that provides novel data and helps to comprehend the system of study.

Mouse models of allergic asthma

Animal models allow for the testing of hypotheses that would not be ethical or practical in humans. For example, the study of the initiation of a disease requires either anticipating a pathology, which is very rare in humans, or inducing the disease, which is unacceptable in humans. Moreover, single pathologies can be modeled and addressed in mice while it is impossible to separate pathologies in humans. The investigation of slow emerging and chronic processes, such as tissue remodeling in allergic asthma or aging, greatly benefit from animal models as these represent a compression in time of a protracted process.

Animal models of allergic asthma reproduce many features of the human disease and, therefore, provide a useful tool for investigating disease initiation and progression, as well as identifying and evaluating novel therapeutic targets. Indeed, much of our current understanding of responses to allergens comes from studies in a variety of laboratory animals. The mouse is the most widely used species because of the many genetic, physiologic and immunologic similarities with humans, availability of numerous inbred and transgenic animals, lower cost and space requirements, short gestational period, easy breeding and the wide array of specific immunological reagents (57). In the experiments included in this thesis, we used allergen exposure models in mice. We primarily used female mice of the BALB/c strain and, occasionally, C57BL/6 mice. Interestingly, these two strains of mice have been traditionally considered to favor either Th2 (BALB/c) or Th1 responses (C57BL/6). However, we and others have shown that they can both develop robust Th2-polarized immune responses to allergens (58). For instance, C57BL/6 mice exhibit higher levels of antigen-specific IgE and eosinophil infiltration into the lungs in response to allergens, compared to BALB/c mice. Most commonly used mouse strains are congenic, thus facilitating the interpretation of results. As humans are genetically heterogeneous, the use of different mouse strains may contribute to a better understanding of the human variability in responses.

Mice do not spontaneously develop asthma. Therefore, a disease-like reaction has to be induced in the airways. A detailed elaboration on experimental models of allergic asthma is included as Appendix I (59). Ovalbumin (OVA) derived from chicken egg is the most commonly used surrogate allergen. OVA is an innocuous protein and exposure, either intranasally (i.n.) or by aerosol, to naïve mice elicits tolerance rather than allergic sensitization or airways inflammation (60-62). Tolerance, understood as a state of specific and intended non-reactivity of the immune system to antigens, is of immense importance for the host as we are continuously exposed to self and exogenous harmless antigens. lmmunologically, this state arises when DCs present Ag to lymphocytes in the absence of appropriate co-stimulatory signals (62-64), thus rendering Ag-specific lymphocytes anergic (non-responsive); alternatively, it may result in their outright deletion from the lymphocyte pool. The typical strategy to circumvent inhalation tolerance involves the administration of OVA adsorbed to a Th2-skewing chemical adjuvant (usually aluminum-based). Indeed, injection of this mix into the peritoneal cavity generates allergic sensitization, and subsequent airway challenge with OVA elicits an asthma-like phenotype, including consistent and robust Th2 inflammation with tissue remodeling and AHR (65-67). Until recently, the vast majority of the research in this field has been performed in these models, commonly referred to as “conventional” models. However, sensitization in humans does not involve the manipulations (route and adjuvant) employed in these models, thus limiting the proper study of this phenomenon. Moreover, OVA is seldom implicated in human asthma. Consequently, alternative allergens with greater clinical relevance are being increasingly used. Environmental antigens that can elicit allergic asthma in humans comprise both indoor and outdoor allergens, including substances derived from animal dander (cats, dogs and rodents), arthropods (such as mites and cockroaches), seasonal pollens and certain molds.

Over ten years ago, our laboratory embarked on a process to develop, for the first time, mouse models of allergic airway disease that employed common environmental allergens and established sensitization by exposure to the respiratory mucosa without additional chemical and exogenous adjuvants. We demonstrated that ten consecutive days of respiratory exposure to a HDM extract was sufficient to elicit

long-lived Th2-polarized sensitization, eosinophilic airways inflammation and AHR (68). We also developed a chronic HDM model of continuous HDM exposure for five weeks that, in addition to Th2 immunity, generated marked airway remodeling and profound AHR (69). These models were instrumental to the work presented in this thesis.

HDM as a model allergen

Dust mites belong to the phylum *Arthropoda*, class *Arachnida*, and subclass *Acarina*. They were described as the source of house dust allergen in 1964 (70). They are the most common indoor aeroallergen source worldwide and 50-85% of asthmatics are allergic to HDM (71). Dust mites are microscopic in size (up to 0.4 mm long and 0.3 mm wide), perennial and pervasive; they thrive in humid and warm environments and are primarily found on carpets, mattresses and soft furnishings. The most common species are *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, which co-exist worldwide although *D. pteronyssinus* is much more prevalent in coastal regions and countries such as UK, Australia and New Zealand (72,73).

The allergenic potential rests with the mites and their fecal pellets, which contain a complex mixture of many protein and non-protein components of various sizes and biological functions (74,75). Appendix I includes a book chapter with an extended section dedicated to mite allergens (59). Briefly, within the allergenic proteins, groups 1, 3, 6 and 9 are essentially proteolytic enzymes (cysteine and serine proteases), which likely play a digestive function in mites, and have been shown to disrupt tight junctions and activate epithelial protease-activated receptors to facilitate leukocyte infiltration, cell degranulation, collagen production and smooth muscle contraction. Group 2, 5, 7, 13, 14 and 21 allergens are fatty acid/lipid-binding proteins, some being similar to proteins within the toll-like receptor (TLR) pathway and, therefore, able to interact with the innate immune system, such as the allergen Der p 2 (MD2-like allergen) facilitating TLR4 complex aggregation. There are other HDM allergens that display non-proteolytic enzymatic activities: group 4, 8 and 20 allergens are amylases, glutathione-S-transferases and arginine kinases, respectively,

whereas groups 12, 15 and 18 display homologies with chitinases. The muscle-derived proteins tropomyosin and paramyosin constitute the HDM allergen groups 10 and 11, respectively. Groups 16 and 17 were identified as gelsolin-like and EF-hand $Ca2^{+}$-binding proteins. HDM is not only an allergen carrier but also an important transporter of endosymbiotic and/or contaminating microbial PAMPs able to trigger innate immunity. For example, mites are in contact with fungi and bacteria and, therefore, rich in microbial compounds, such as lipopolysaccharide (LPS) and β-glucans (76). Chitin, a potent adjuvant able to polarize Th1, Th2 and Th17 immune responses, is present in the fungi cell wall and also constitutes the mite exoskeleton and is found in the faeces (77).

Commercial HDM extracts vary largely according to their preparation, resulting in markedly different biochemical properties, such as endotoxin content (78). In all studies of this thesis, a commercially available HDM extract from Greer® consisting of crushed and lyophilized body mites and fecal pellets from *D. Pteronyssinus* was used. A recent publication showed that, compared to other extracts, the Greer® extract has very low serine/cysteine protease activity but induces the greatest effects on epithelial barrier function, including delocalization of junctional proteins and significant increases in chemotactic factors. These changes are accompanied by the most pronounced induction of HDM-specific IgE, goblet cell hyperplasia, IL-5, eosinophilic inflammation and AHR (78). The Greer® extracts that we used were rich in proteins (~1 mg/ml) and low in LPS (~25-100 EU/ml), which corresponds to 1 ng LPS/mouse/day at most (when giving 25 μg HDM/day), an amount that cannot induce *per se* an inflammatory response in the lung. Indeed, 10 ng LPS (given intratracheally (i.t.)) was shown to slightly elevate IL-6 and tumor necrosis factor α (TNF-α) in bronchoalveolar lavage (BAL) (79) and the neutrophil response threshold was reported to be 300 ng LPS (given i.n.) (80). In a study using OVA depleted of contaminating LPS (<1 ng) as antigen, low doses of inhaled LPS (0.1 μg) promoted Th2 responses to OVA, with high IgE and eosinophilic inflammation, whereas high doses of LPS (100 μg) induced protective Th1 responses through down-regulation of Th2-type cytokines, prevention of eosinophilic inflammation, mucus and AHR but induction of neutrophil recruitment and IgG2a (81). In short, the extracts of HDM used in the

experiments of this thesis had very low to negligible amounts of LPS and, therefore, any observed responses were primarily mediated by other HDM components.

Environment-host interactions

Factors influencing the development of allergic asthma

That allergic asthma occurs in susceptible individuals implies that the state of the host prior to allergen exposure can influence the onset, nature and progression of disease. In this regard, family history is considered a risk factor, since a person with asthmatic parents is 3 to 6 times more likely to develop asthma (82). These studies, though, do not definitively delineate genetic from environmental risks because of shared environments in families. Much effort has been dedicated to identify asthma susceptibility genes. Indeed, human polymorphisms in over a hundred genes have been associated with different features of the asthmatic phenotype (83). However, each gene generally has a very low attributable risk and there is poor replication across different populations. Also, the reported statistical associations do not imply causal relations. Lastly, the dramatic increase in asthma prevalence over a relatively short period of time argues against a population-wide genetic shift, and favors an integrated multi-factorial paradigm that takes into account not only the genetic background but also the effects of environmental and lifestyle factors.

It is well known that multiple environmental factors augment the asthmatic response in an already sensitized individual. In addition, there is evidence that some of these factors may also facilitate *de novo* allergic sensitization leading to airway inflammation and lung dysfunction. The Hygiene Hypothesis, proposed by Strachan in 1989 (84), is a prominent theory about the origins of allergic asthma. It purports that improved hygiene in industrialized societies, with improved public health measures and the widespread use of vaccines and antibiotics, has reduced exposure to infectious agents (viruses and bacteria), commensal microorganisms and parasites that would normally stimulate the immune system and mitigate against asthma and allergies (75,85-87). The most popular proposed mechanism of action claims that insufficient stimulation of Th1 immunity, due to reduced microbial exposure, leads to

an overactive Th2 immune response. However, the recent rise in Th1-mediated autoimmune diseases, such as inflammatory bowel disease and type I diabetes, seems paradoxical if the hypothesis is approached solely from a Th1 versus Th2 perspective. An alternative explanation is that the developing immune system must receive microbial stimuli in order to adequately develop immune-regulatory mechanisms, such as regulatory T cells, able to repress both Th1 and Th2 immunity and control both autoimmune and allergic responses (85,87). Importantly, there has been a considerable amount of controversy regarding the impact of infections on the development of allergic asthma. While an interpretation of the Hygiene Hypothesis would argue that viral and bacterial exposure have a protective effect, studies have emerged showing that certain infections in early life may, in fact, promote the development of asthma (88-91). In this regard, it is increasingly apparent that the timing, type and amount of microbial exposure can influence the outcome. For example, the effects of endotoxin are dose-dependent and exposure early in life, but not later, seems to protect, as shown in both animal models and studies in humans in contact with farming environments (86). Finally, other factors may also explain the increase in allergies, including exposure to certain pollutants (diesel exhaust, particulate matter, tobacco smoke, occupational chemicals, pool chlorine) and certain diet/lifestyles (92). Asthma is, therefore, considered a multifactorial disease. In this context, the challenge is to elucidate the immune mechanisms involved and to determine the extent of exposure that will ensure safety.

The airway epithelium and allergic asthma

The respiratory epithelium is the lining of the airway, extending from the nasal cavity through the branching respiratory tree (including the trachea, bronchi, bronchioles and smaller airways) to the terminal air sacs of the lungs. Therefore, lung epithelial cells (ECs) lie at the interface between the host and the environment and represent the first line of defense against inhaled microorganisms, particles, gases and allergens. ECs express many pattern recognition receptors to rapidly detect and respond to environmental stimuli by secreting endogenous signals, including

cytokines, chemokines and antimicrobial peptides, thereby attracting and activating innate and adaptive immune cells (93).

Of particular interest to this thesis (Chapter 4) is the cytokine and growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF). Among other functions, GM-CSF is one of the most powerful natural cytokines that stimulates the accumulation, proliferation and maturation of APCs, such as DCs and macrophages, and enhances the survival and activation of eosinophils and T cells. Details about the structure and immunobiologic functions of GM-CSF and its family members IL-3 and IL-5 are included in Appendix II (53). Increased levels of GM-CSF have been found in human asthmatic bronchial epithelium, BAL and sputum (94-97) and are generally correlated with disease severity (98,99). Moreover, polymorphisms of the *gm-csf* gene are associated with atopy and asthma (100). Our laboratory showed that exogenous administration of GM-CSF in the airways of mice facilitates allergic sensitization and eosinophilic inflammation to OVA, and enhances responses to ragweed (101-103). In addition, we reported that allergic responses to HDM are in part mediated by endogenous GM-CSF production (68). In Chapter 4, we extend these findings on the role of GM-CSF in allergic disease in an experimental model of GM-CSF-induced responses to sub-threshold amounts of HDM.

Over the last few years, there has been intense interest in the role of the EC-associated cytokines thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 on the development of Th2 responses at mucosal sites (36,104). These cytokines share little to no structural homology, and have diverse cellular sources as well as both innate and adaptive cell targets. TSLP is a member of the IL-2 cytokine family and a distant paralog of IL-7, and the high-affinity TSLP receptor (TSLPR) complex includes the TSLPR subunit and the IL-7Rα chain (105); IL-25 (*a.k.a.* IL-17E) signals through IL-17 receptor B (IL-17RB) and IL-17RA (106); IL-33 is an IL-1 family member and mediates its biological effects via interaction with the receptors ST2 (*a.k.a.* T1) and IL-1R accessory protein (IL-1RAcP) (107). A commonality of TSLP, IL-25 and IL-33 is that they all influence APCs, primarily DCs, to establish conditions for naïve $CD4^+$ T cells to differentiate into Th2 cells, including increased expression of MHC II and the co-stimulatory molecule OX40L, secretion of Th2 cell-attracting chemokines and/or

decreased production of IL-12/23p40 (36,104). In mice, transgenic overexpression or delivery of TSLP, IL-25 or IL-33 leads to marked increases in Th2 cytokines, IgE, lung eosinophilic inflammation, goblet cell hyperplasia and AHR (43,108-111). Moreover, recent reports have shown that IL-25 and IL-33 can expand ILCs able to produce Th2 cytokines (112). Clinical studies have revealed higher TSLP, IL-25 and IL-33 expression in the lung of asthmatics compared to healthy subjects, with a particular association with severe asthma (108,113-116); and genetic analyses have linked polymorphisms in the genes encoding for TSLP, IL-17RB, IL-33 and ST2 with allergic disease and asthma (117-119). Importantly, most experimental studies supporting a role for these cytokines in the generation of allergic respiratory responses have relied on conventional OVA models (120-122). The use of blockade or knockout (KO) strategies to prevent TSLP and IL-25 signaling unanimously shows that these cytokines are critical (110,120,121). However, the precise relevance of IL-33 signaling remains unclear given discrepancies in different asthma models and blocking strategies. Blockade of ST2 or IL-33 has been shown to abrogate Th2 cytokine production, eosinophilic inflammation, mucus production, IgE and AHR in murine OVA models (122-125). In contrast, other groups using ST2 KO mice have reported normal OVA-induced inflammation and eosinophilia (126,127). In Chapter 3, we investigated the involvement of this triad of cytokines in the generation of allergic sensitization to inhaled HDM.

Overall, a model is emerging whereby communication between ECs and DCs, likely via the aforementioned cytokines, determines the outcome of the immune response (93,128). Interestingly, there is evidence to support a degree of cross-regulation between these EC-associated cytokines, although the complexities of these inter-relationships have not yet been clearly defined. Chapter 4 provides novel data in this regard.

Different types of respiratory ECs line the airways, each uniquely suited to its location and function. For example, the epithelium of conducting airways consists of ciliated, goblet (mucus-producing) and basal cells situated on top of the basement membrane. Of interest to Chapter 4 are the alveolar cells found in the pulmonary alveoli. In the wall of these air sacs, there are two cell types located in single layers:

type I alveolar cells are squamous, large flattened cells that conduct gas exchange; and type II alveolar cells (ATII), also termed type II pneumocytes, are cuboidal (shorter and fatter) and secrete surfactant. Surfactant is a lipid and protein complex that coats the alveolar walls to lower the surface tension, prevents alveoli from collapsing during exhalation and decreases the energy required to inflate the lungs. ATII cells are capable of proliferating for self-renewal and can transdifferentiate into type I cells, repairing the injured epithelium (129,130). In addition, both human and murine ATII cells have been found to express TLRs, respond to cytokines and secrete a variety of immune cell modulatory factors, including antimicrobial peptides, components of the complement pathway, cytokines and chemokines (131,132). ATII cells can release and respond to GM-CSF, which has been shown to mediate hyperplasia and hypertrophy on ATII cells (133,134). Furthermore, recent studies using IL-33 reporter mice have found that ATII cells are the major source of IL-33 at baseline and upon allergic lung inflammation following exposure to OVA, fungus, ragweed or papain (135,136). The involvement of ATII cells during sensitization to HDM remains unknown.

Central aim and hypotheses

The fundamental goal of immunity is to protect the host against harmful intruders and prevent disease. From this perspective, there is no apparent survival advantage in generating a productive immune-inflammatory response against seemingly harmless antigens, such as HDM allergens. Despite remarkable progress in our understanding of the pathogenesis of allergic asthma, the answer to the question "*why do some people become allergic and develop asthma whereas others do not?*" remains elusive. This thesis investigates the ontogeny of allergic asthma, providing novel insights into both environmental conditions and key cellular and molecular players influencing the development of the disease.

Aeroallergen exposure is a prerequisite for the development of allergic asthma. However, less is known about what (the type and presentation of allergen), when (the age of exposure), how (the route of exposure), how much (the dose of exposure) and for how long (the duration of exposure) allergen exposure may cause asthma, be protective, or have no effect in some, but not other, individuals. In Chapter 2 of this

thesis, we hypothesized that the *amount* of HDM and the *duration* of exposure could influence the development and severity of allergic responses. To this end, a dose-escalation regimen was used to determine the sensitization and elicitation thresholds on mice. Moreover, the evolution of responses was visualized over time, uncovering non-linear relationships between HDM exposure and either allergic sensitization or airway inflammation.

As mentioned earlier, the airway epithelium is currently viewed not only as a protective barrier in constant contact with environmental agents, such as allergens, but also as a bridge between innate and adaptive immunity. In this regard, evidence supports a pivotal role of *EC-derived molecules* in triggering allergic asthma. In Chapter 3, we hypothesized that HDM-induced production of EC-associated cytokines could activate DCs and promote allergic sensitization. Our data showed that HDM upregulated TSLP, IL-25 and IL-33 but only IL-33 was critical for intact allergic sensitization likely via upregulation of OX40L on DCs and expansion of type 2 innate lymphoid cells.

Ample experimental evidence suggests that modulation of DC functions can determine the type and extent of responses to allergens, primarily due to the way DCs interact with, and regulate, T cells. In Chapter 4, we hypothesized that environmental agents able to alter lung DCs could facilitate sensitization to amounts of HDM that would otherwise not induce an allergic response. We show that a *GM-CSF-enriched lung environment*, a common scenario upon exposure to infectious agents and pollution among others, induced IL-33 production and activated DCs, thus facilitating responses to sub-threshold amounts of HDM. This highlights the importance of concomitant environmental exposures on the dose of allergen causing sensitization.

Collectively, these three studies demonstrate that there is an intimate relationship between environmental immune stimulation and the development of disease. The work in this thesis provides novel mechanistic insights into how asthma is initiated, thus furnishing a framework to explore new intervention strategies that could prevent and/or alter the natural course of the disease.

Chapter 2.

In Vivo-to-*In Silico* Iterations to Investigate Aeroallergen-Host Interactions

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In Vivo-to-*In Silico* Iterations to Investigate Aeroallergen-Host Interactions

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Abstract

Background: Allergic asthma is a complex process arising out of the interaction between the immune system and aeroallergens. Yet, the relationship between aeroallergen exposure, allergic sensitization and disease remains unclear. This knowledge is essential to gain further insight into the origin and evolution of allergic diseases. The objective of this research is to develop a computational view of the interaction between aeroallergens and the host by investigating the impact of *dose* and *length* of aeroallergen exposure on allergic sensitization and allergic disease outcomes, mainly airway inflammation and to a lesser extent lung dysfunction and airway remodeling.

Methods and Principal Findings: BALB/C mice were exposed intranasally to a range of concentrations of the most pervasive aeroallergen worldwide, house dust mite (HDM), for up to a quarter of their lifespan (20 weeks). Actual biological data delineating the kinetics, nature and extent of responses for local (airway inflammation) and systemic (HDM-specific immunoglobulins) events were obtained. Mathematical equations for each outcome were developed, evaluated, refined through several iterations involving *in vivo* experimentation, and validated. The models accurately predicted the original biological data and simulated an extensive array of previously unknown responses, eliciting two- and three-dimensional models. Our data demonstrate the non-linearity of the relationship between aeroallergen exposure and either allergic sensitization or airway inflammation, identify thresholds, behaviours and maximal responsiveness for each outcome, and examine inter-variable relationships.

Conclusions: This research provides a novel way to visualize allergic responses *in vivo* and establishes a basic experimental platform upon which additional variables and perturbations can be incorporated into the system.

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Introduction

Allergic asthma emerges from the interaction between two complex dynamic systems, the immune system and the environment, where aeroallergens exist. These systems are intricate, comprise multiple parts which are subject to many interactions and feedback loops and, consequently, contain a broad array of outputs. The interaction between these already complex systems generates an even higher degree of complexity. Thus, deciphering the conditions under which allergic disease evolves would benefit from the elaboration of models that can explain and/or predict the potential outputs of that interaction.

Advances in the understanding of disease processes have come in great measure through experimentation using *in vitro* and, notably, *in vivo* human and animal models. A detailed appreciation of the immunopathology of asthma, along with the explosion in molecular immunology has prescribed the modeling strategies to recapitulate the asthmatic phenotype, particularly in mice. It should be noted that conventional biomedical modeling greatly differs from modeling in other scientific domains, such as ecology or economics in that biomedical models are conceived with a pre-established goal in mind: to establish a known phenotype. While such an approach has produced conspicuous benefits, it has inherently prevented an unbiased, global understanding of the consequences of the interaction between allergens and the immune system.

Although it is generally thought that there is a reasonable correlation between early allergen exposure and sensitization [1,2,3,4] or sensitization and disease [1,5,6], the connection that may exist between exposure and disease is less clear [1]. The intrinsic constraints of these clinical and epidemiological studies

preclude achieving both a longitudinal and quantitative understanding of these relationships. Yet, it seems intuitive that such knowledge is essential to gain further insight into the origin, evolution and nature of allergic disease.

The strategy that we followed to investigate the relationship between aeroallergen exposure, allergic sensitization and allergic disease embraces a computational conception of immune responsiveness [7]. In this conception, the view is *synthetic* rather than *analytical* and, therefore, the focus is on system behaviors rather than specific components, i.e. the complex molecular networks underlying the outcomes that we measured. We surmise that this strategy is justified *ad interim* given the current state of knowledge in systems biology *in vivo*. We present data delineating the kinetics and dose-responses for local (total inflammation and eosinophilia) and systemic (HDM-specific immunoglobulins (Ig) G_1 and E) events elicited in mice by extended exposure to house dust mite (HDM). We developed and refined algorithms defining the behavior of each outcome that were subsequently used to conduct *in silico* simulations to guide new biological experiments and visualize an extensive array of unknown responses. We propose that the iterative approach applied to construct the model exhibits considerable fidelity to the biological structure of the process.

Methods

Animals

Female BALB/C mice (6 to 8 weeks old) were purchased from Charles River Laboratories. The mice were housed in a specific pathogen-free environment under 12 h light-dark cycle. All experiments described in this report were approved by the Animal Research Ethics Board of McMaster University.

Protocol of respiratory mucosal sensitization

House dust mite extract (Greer Laboratories) was resuspended in saline (0.9% NaCl Irrigation Solution, Baxter) and serial dilutions were done to obtain the desired concentrations. This suspension was delivered to isoflurane-anaesthetized mice intranasally in a 10 μl volume. Mice were exposed daily to HDM for either 10 consecutive days (short-term protocol) or 5 consecutive days a week followed by 2 days of rest for a total of 1, 2, 3, 5, 7, 10, 14 and 20 weeks (long-term protocol).

Sample collection

At various time-points, always 72 hours after the last HDM exposure, mice were sacrificed. Blood was collected by retro-orbital bleeding. Blood smears where prepared and serum was obtained by centrifugation of whole blood. Bronchoalveolar lavage (BAL) was performed as previously described [8,9]. Briefly, the lungs were dissected, the trachea was cannulated with a polyethylene tube (BD Biosciences) and two lavages were done with PBS (0.25 ml followed by 0.2 ml). Total cell counts were then determined using a hemocytometer and smears were prepared by cytocentrifugation. Protocol Hema 3 stain set (Fisher Scientific) was used to stain blood and BAL smears and differential cell counts (≥500 leukocytes) were determined according to a previously established protocol [9]. The right lobe of the lung was inflated and fixed in 10% formalin for histological analysis.

HDM-specific Ig measurements

Levels of HDM-specific IgE and IgG_1 in serum were measured using ELISA techniques as previously described in detail [10]. Optical density (OD) was read at 405 nm. HDM-specific IgE titres (in OD units) were calculated by subtracting from each sample OD the average OD value of 20 zero standard replicates plus two standard deviations. HDM-specific IgG1 titers (in relative units) were calculated using the formula $1/(x/ODx*0.05)$, where x equals the dilution factor closest to but greater than double the average OD value of 20 zero standard replicates, and ODx is the OD reading of x.

Determination of airway responsiveness

Mice were anesthetized with nebulized isoflurane (3% with 1 L/min of O_2), paralyzed with pancuronium bromide (1 μg i.p.), tracheostomized with a blunted 18-gauge needle, and mechanically ventilated with a small animal computer-controlled piston ventilator (flexiVent, SCIREQ Inc.) [11]. Mice received 200 breaths per minute and a tidal volume of 0.25 ml; the respiratory rate was slowed during nebulization (10 seconds) to provide 5 large breaths of aerosol at a tidal volume of 0.8 ml. The response to nebulized saline and increasing doses (3.125, 12.5 and 50 mg/ml) of methacholine (MCh, Sigma-Aldrich) was measured. A positive-end-expiratory pressure of 3 cm of H_2O was applied by submerging the expiratory line in water. Respiratory impedance was determined from 3 second broadband volume perturbations ranging from 1 to 20.5 Hz every 10 seconds during approximately 2 minutes following each dose of MCh. The data was fitted with the constant phase model and model parameters (airway resistance (Rn), tissue dampening (G), tissue elastance (H) and hysteresivity, a measure of lung heterogeneity ($\eta = G/H$)) were calculated [12]. Model fits that resulted in a coefficient of determination less than 0.8 were excluded.

Histology and morphometric analysis

Lung tissue was embedded in paraffin and cut at a thickness of 3 μm. Sections were stained with hematoxylin and eosin for evaluation of the severity and the nature of leukocyte infiltration in the lungs by light microscopy. Additional sections were stained with Picro Sirius red to demonstrate the presence of collagen in the extracellular matrix. Images of main airways were captured with OpenLab (Improvision) via a Leica camera and microscope attached to a computer. Analysis was performed on a custom computerized image analysis system (Northern Eclipse software, Empix Imaging) as previously described [13]. Briefly, morphometric quantification involved calculation of the percentage of tissue area that was positively stained within a 40 μm-thick area from the basement membrane extending into the airway lumen.

Mathematical and computational modeling

All the equations for the mathematical models and analyses were generated using curve-fitting techniques within FindGraph software (UNIPHIZ Lab) for each outcome. (All equations and additional detail on the validation analysis of the model is provided in an online data supplement)

Data analysis

Data are expressed as means±standard error of the mean (s.e.m.). Statistical analysis was performed with GraphPad Prism (GraphPad Software). Results were interpreted by analysis of variance (one-way ANOVA) followed by the Dunnett *post hoc* test to compare HDM exposed groups versus the saline control group. Differences were considered statistically significant when p values where less than 0.05.

Results

Dose-response to short-term HDM exposure

We have previously shown that mice exposed intranasally to HDM at a concentration of 25 μg/day for 10 consecutive days develop acute airway inflammation [14], and that exposure to 25 μg/day for up to 7 weeks establishes chronic airway

inflammation associated with remodeling [15]. These static conditions, fixed times and concentrations, were selected to achieve desired specific outcomes, thus neglecting the dynamic nature of a living system. Hence, we initially carried out a dose-response experiment using a 10,000-fold range, from 0.01 to 100 μg/day, for 10 days. As shown in Figure 1A, the response in terms of total cell numbers (TCN), eosinophils (EOS) and HDM-specific serum IgG_1 followed a logistic pattern with an incipient response observed with 1 μg and a plateau after 25 μg. Based upon these findings, we chose doses of 1 (incipient), 7.5 (moderate) and 25 μg/day (submaximal) for subsequent experiments utilizing longer exposure periods.

Modeling the inflammatory response to long-term HDM exposure

We investigated the impact of exposing mice to those three concentrations of HDM for up to 14 weeks. As shown in Figure 2A, repeated allergen exposure initially elicited airway inflammation in a near-exponential manner that was both dose- and time-dependent. At 2 weeks, 7.5 and 25 μg led to a distinct peak in inflammation; from then on, 25 μg maintained a stable level of maximal inflammation. A similar plateau was also achieved with 7.5 μg only after 7 weeks. Interestingly, exposure to 1 μg of HDM even for such a protracted period of time did not elicit significant airway inflammation suggesting that a threshold of responsiveness for this outcome must be above this concentration.

Once experimental data were collected and analyzed, a *bottom-up* model was constructed using mathematical and computational methods to accurately portray the data and the ensuing dynamics. Although identical methodology was used for all outcomes (TCN, EOS and immunoglobulins), detailed steps (Figure 1B) are presented for only TCN for brevity.

The initial equation was encoded to be used iteratively to simulate and predict output responses given inputs of dose and time. Figure 2B shows a *retrodiction* of the model in which simulations were compared to actual data and proved to fit fairly well. Given that lower doses (saline and 1 μg) exhibited a seemingly different behavior compared to higher doses (7.5 and 25 μg), an intermediate dose of 5 μg was selected to further assess the model. In this evaluation, a *prediction*, or interpolation, of a 5 μg dose was performed and compared to actual data (Figure S1A). Figure 2C depicts a refined model that incorporates these new experimental data.

An additional evaluation of the model was also carried out by extrapolating data up to 20 weeks (Figure S1B). Following comparison to actual data, the model was further refined to include all experimental data (Figure 2D), resulting in a final equation generated from 5 doses and 9 time-points. Equation 1 describes total inflammation (y) as a function of time (t) and dose (x), while each of the respective co-efficients (x_{a1}, x_{b1}…) represent dose-dependent quantities (see Methods S1, equations 1.1 to 1.21):

$$y = f_C^{TCN}(x,t) = \begin{cases} x_{a1} + x_{b1}\sin(z_1(x,t))^2 & , \quad 0 \le t \le 3 \\ x_{a2} + x_{b2}e^{z_2(x,t)} + x_{g2}t + x_{h2}t^2 & , \quad 3 \le t \le 7 \\ x_{a3} + x_{b3}e^{z_3(x,t)} + x_{g3}e^{z_4(x,t)} & , \quad t \ge 7 \end{cases} \quad (1)$$

While the model visually fit the experimental data, accuracy was verified and quantified mathematically. Using linear regression, the initial equation, the revised equation (including the 5 μg data), and the final equation (including both the 5 μg and 20 week data) yielded R^2 values of 0.987, 0.987 (not shown), and 0.990, respectively (Figure 2E). Furthermore, 95% confidence intervals (CI) and global validation metrics were calculated (see online data supplement and Figure 2F). The latter accounts for experimental uncertainty, error and chance [16,17,18], and confirmed that not only the model accurately predicts actual responses but also that an additional dose in the model did not enhance the accuracy of the system, while the integration of a further time-point had only a minimal effect.

This *complex* model accurately mimics varying system dynamics. However, in order to facilitate the visualization of responses, we developed a *simple* model (see online data supplement), represented by Equation 2, which captures the general features of the system:

$$y = f_S^{TCN}(x,t) = \frac{x_a + x_b t + x_c t^2}{1 + x_d t + x_g t^2} \quad (2)$$

To visualize the dynamics of the system, simulations were performed with both *complex* and *simple* models to emulate the response between 0 and 25 μg, in sequential 0.5 μg increments over a 20 week period (Figure 3A–B). Further extrapolations were performed doubling both the highest dose and latest time-point used to construct the model. Figure 3C shows simulations in three-dimensions up to 40 weeks (approximately 50% of the lifespan of a

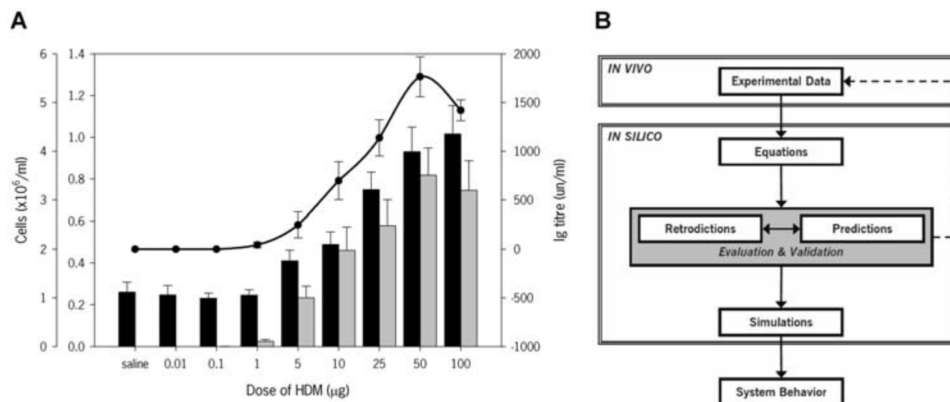

Figure 1. Airway inflammation and systemic immunity in BALB/C mice exposed to HDM for 10 consecutive days. (**A**) Dose-response: total inflammation (black bars), eosinophilia (grey bars) and serum HDM-specific IgG_1 (solid circles and solid line). Results for cells (n = 5–19 mice/group) and IgG_1 (n = 2–6 mice/group) are expressed as means±s.e.m. (**B**) Schematic of the steps followed to develop the mathematical models.
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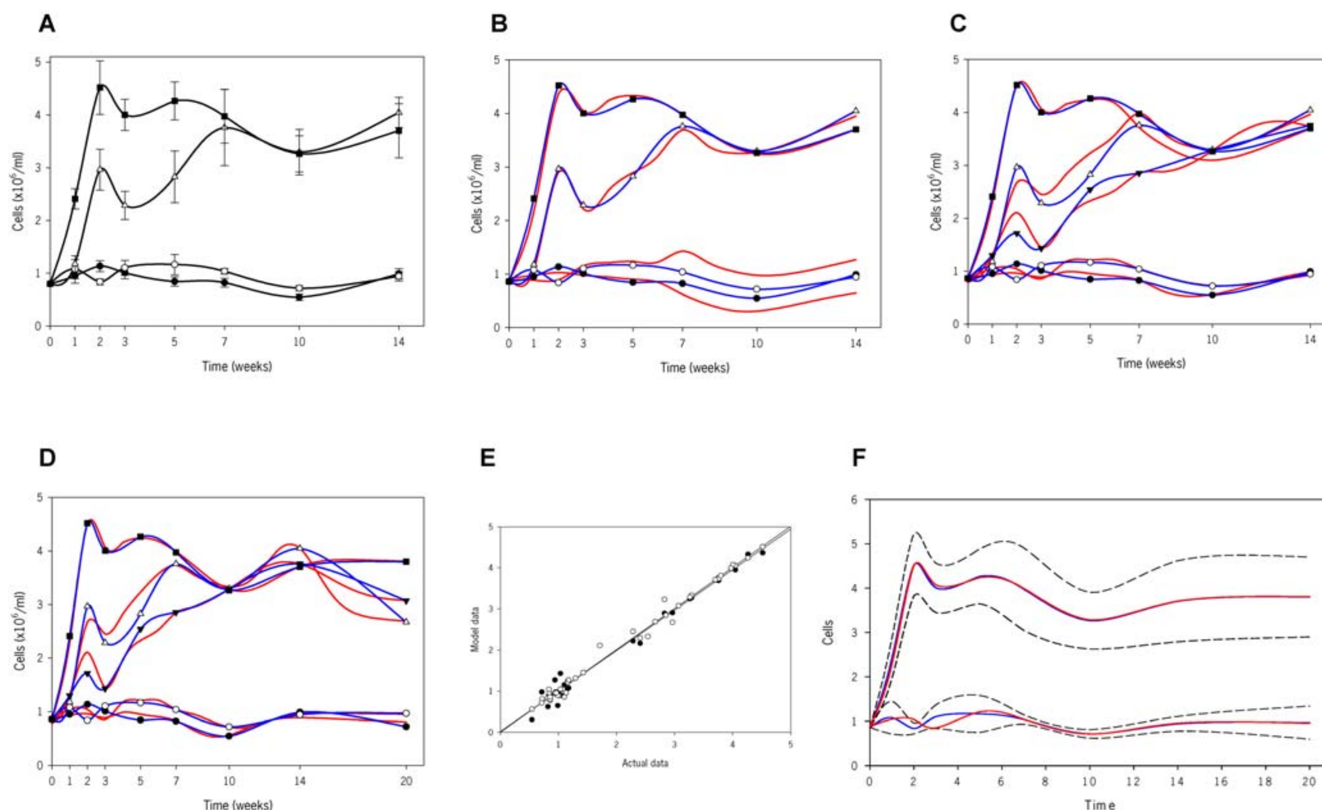

Figure 2. Airway inflammation in BALB/C mice exposed to HDM and subsequent mathematical modeling. (**A**) Inflammatory response in the BAL. Mice were exposed intranasally to either saline (solid circles) or HDM, 1 μg (open circles), 7.5 μg (open triangles up) or 25 μg (solid squares) for up to 14 weeks (5 days of exposure and 2 days of rest per week). Cell numbers are expressed as mean±s.e.m (n = 6–12 mice/group). (**B**) Mathematical modeling of the inflammatory response. A mathematical equation was developed from the experimental results (blue lines) based on dose and length of exposure to HDM. Simulations (red lines) for each of the doses studied experimentally were generated. (**C**) Results of the first iteration. Predictions using the first mathematical model were generated for 5 μg of HDM (see Figure S1) and were subsequently evaluated experimentally. Then, the new 5 μg experimental data (solid triangles down) was used to readjust the previous equation and refine the model. Actual data (blue lines) and virtual simulations (red lines) are shown for saline, 1, 5, 7.5 and 25 μg of HDM up to 14 weeks. (**D**) Results of the second iteration. Predictions using the second mathematical model were generated for all doses studied at 20 weeks of exposure (not shown) and were subsequently evaluated experimentally. Then, the new 20 week data was used to readjust the previous equation and further refine the model. Actual data (blue lines) and virtual simulations (red lines) are shown for saline, 1, 5, 7.5 and 25 μg of HDM up to 20 weeks. (**E**) Regression analysis to evaluate the accuracy of the mathematical models. Deterministic validation metrics were performed to mathematically measure the agreement between computational predictions and experimental results. For the line y = x, where y is model data and x is actual data, the coefficient of determination R^2 in the first model is 0.987 (solid circles) and 0.990 in the last one (open circles). (**F**) Confidence intervals (CI) to evaluate the accuracy of the mathematical models. Non-deterministic validation metrics were also used to account for experimental and computational uncertainties and errors. The 95% CI for each point depicts uncertainty due to experimental variability. Model data for 25 μg (top) and 1 μg (bottom) accurately predict actual data, and fall within the 95% CI band.
doi:10.1371/journal.pone.0002426.g002

mouse) and 50 μg to further enhance the visualization of the dose-time-response relationship. The *complex* model predicts actual data with slightly greater accuracy than the *simple* model, as indicated by an approximate 0.05 increase in the R^2 value and an 8.5% increase in the 95% CI (data not shown and Figure 3D). Such simulations epitomize visual and numerical information that can only be derived mathematically and computationally.

Analysis of global inflammation was followed by an evaluation of airway eosinophilia, a typical hallmark of allergic inflammation. As illustrated in Figure 4A, eosinophils initially increase in a dose- and time-dependent fashion but later dramatically decrease to a level of 6–9% of total cells at 20 weeks of exposure. These findings are supported by a histopathological assessment (Figure 4E). As shown in the top panels, inflammation at 2 weeks is minimal in mice exposed to 1 μg of HDM and severe in those exposed to 25 μg. A graded level of inflammation was evident after exposure to 5, 7.5 and 25 μg (data not shown). Figure 4E (bottom panels) depicts a comparison of acute versus chronic exposure revealing stable inflammation over time but a relative decrease in tissue eosinophilia at later time-points. To note, we also observed a similar decrease in eosinophils in peripheral blood (data not shown). At variance with these findings, neutrophils and, particularly, mononuclear cells increased throughout the entire duration of allergen exposure, numerically compensating for the decrease of eosinophils and, hence, maintaining the overall degree of inflammation (Figure 4F). Again, exposure to 1 μg of HDM for up to 14 weeks did not elicit any significant changes in eosinophils (Figure 4A), or mononuclear cells and neutrophils (data not shown).

Using the aforementioned methods, *complex* and *simple* models were constructed for eosinophils, eliciting the equations:

$$y = f_C^{EOS}(x,t) = \begin{cases} x_{a1} + x_{b1}e^{z_1(x,t)} + x_{g1}e^{z_2(x,t)} & , \quad 0 \le t \le 7 \\ x_{a2} + x_{b2}t + x_{c2}t^2 + x_{d2}t^3 & , \quad t \ge 7 \end{cases} \tag{3}$$

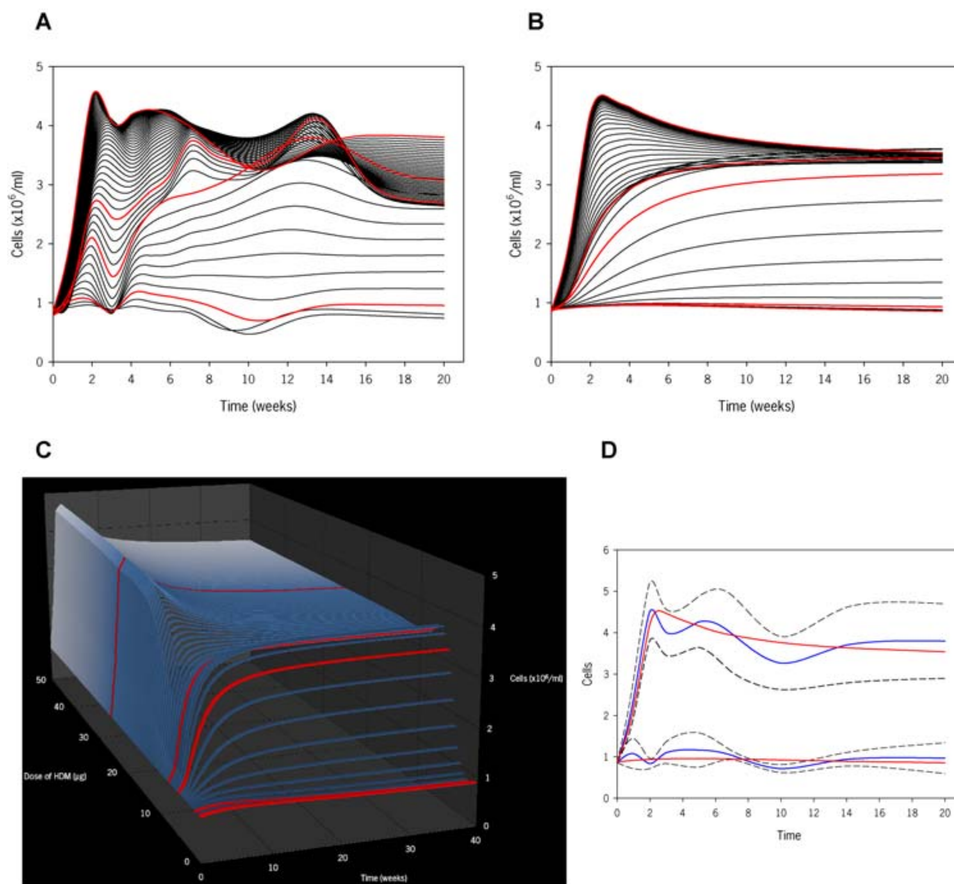

Figure 3. 2D and 3D models for total cells generated from mathematical equations. In panels **A**, **B** and **C**, the simulations for the doses of HDM used experimentally are highlighted in red. (**A**) Simulations based on dose of HDM (range from 0 to 25 µg at 0.5 µg intervals) and length of exposure (0 to 20 weeks) using the final mathematical model (Figure 2D). (**B**) Simplified mathematical model for the total cell number. (**C**) 3D simulations generated from the simplified mathematical model including predictions up to 50 µg and 40 weeks of HDM exposure. (**D**) Confidence intervals to evaluate the accuracy of the simple mathematical model. Visual inspection shows that the simple model falls within the 95% CI, while quantification of the simple TCN model accuracy was calculated to be 89.03±22.42% with 95% confidence. Thus, the simple TCN model has similar fidelity to the complex model.
doi:10.1371/journal.pone.0002426.g003

$$y=f_S^{EOS}(x,t)=\left|\frac{t+x_a}{x_b+x_c(t+x_a)+x_d(t+x_a)^2}\right| \quad (4)$$

Although Equations 3 and 4 represent different functions, they both maintain relatively high and similar predictive value, yielding R^2 values of 0.968 and 0.938, respectively. Using these equations, we performed computer simulations in two- and three-dimensions (Figure 4B–D). These images illustrate that the decrease in airway eosinophilia occurs throughout the entire range of exposures, and that is not dependent on eosinophilia reaching an absolute level; moreover, it is also evident that the higher the eosinophil level, the sooner the downturn begins. This suggests that part of the program of the immune-inflammatory response elicited by chronic allergen exposure may contain an inherent controlling mechanism to prevent persistent eosinophilia in the lung.

Modeling allergic sensitization to long-term HDM exposure

Allergic sensitization is a crucial event in allergic asthma. Hence, we investigated the effect of allergen exposure on defining features of B cell immunity, namely serum levels of HDM-specific immunoglobulins. As shown in Figure 5A–D, IgG_1 and IgE serum levels follow a logistic-like behavior similar to that identified for total airway inflammation. However, there are kinetic differences; indeed, serum immunoglobulins are detected after 2–3 weeks of allergen exposure at a time where the inflammatory response has already reached its peak. Given the nature of the immunoglobulin response, *simple* models for both IgE and IgG_1 were constructed and proved to be accurate (R^2 values of 0.900 and 0.985, respectively). Equations 5 and 6 depict the values of IgE and IgG_1:

$$y=f_S^{IgE}(x,t)=\frac{x_a t^{x_d}}{x_c^{x_d}+t^{x_d}} \quad (5)$$

$$y=f_S^{IgG_1}(x,t)=x_a+\frac{x_b-x_a}{1+\left(\frac{t}{x_c}\right)^{x_d}} \quad (6)$$

Relationship between allergic sensitization and airway inflammation

Airway inflammation and allergic sensitization were compared using two different approaches. First, we considered the *threshold* of

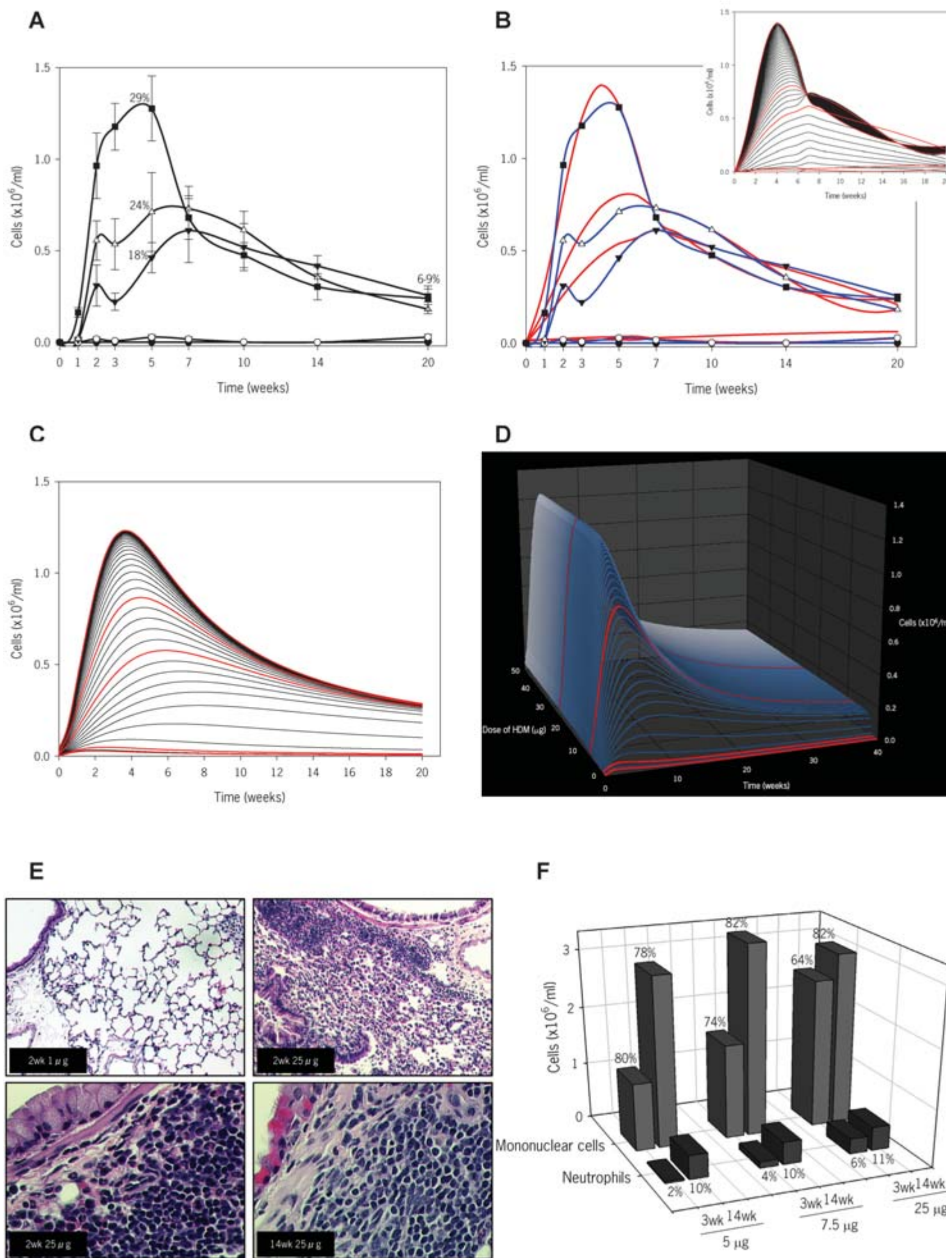

Figure 4. Nature of the inflammatory response in mice exposed to HDM and mathematical modeling of eosinophils. In panels **B** ***insert***, **C** and **D**, the simulations for the doses of HDM used experimentally are highlighted in red. (**A**) Eosinophilic response in the BAL fluid. Mice were exposed to either saline (solid circles) or HDM, 1 μg (open circles), 5 μg (solid triangles down), 7.5 μg (open triangles up) or 25 μg (solid squares) for up to 20 weeks. Eosinophil numbers are expressed as mean±s.e.m (n = 6–12 mice/group); percentage of eosinophils at 5 and 20 weeks are inserted in the graph. (**B**) Final mathematical model for eosinophils. The equation to obtain these predictions (red lines) was developed from the experimental results (blue lines) based on dose and length of exposure to HDM. The ***insert*** shows simulations based on dose of HDM (range from 0 to 25 μg at 0.5 μg intervals) and length of exposure (0 to 20 weeks) using the final mathematical model. (**C**) Simplified mathematical model for eosinophils. (**D**) 3D simulations generated from the simplified mathematical model including predictions up to 50 μg and 40 weeks of HDM exposure. (**E**) Light photomicrograph of lung sections stained with hematoxylin and eosin. Top left: after 2 weeks of exposure to 1 μg of HDM (×10 magnification); top right: after 2 weeks of exposure to 25 μg (×10); bottom left: after 2 weeks of exposure to 25 μg of HDM (×40); bottom right: after 14 weeks of exposure to 25 μg of HDM (×40). (**F**) Cellular profile in the BAL fluid. Absolute numbers and percentage of mononuclear cells (light grey bars) and neutrophils (dark grey bars) after continued exposure to 5, 7.5 and 25 μg of HDM for either 3 or 14 weeks. Bars represent mean of cells (n = 6–12 mice/group).
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responsiveness, understood here as the lowest dose of allergen that elicits a measurable response. To address this, we calculated areas under the curve (AUC) for all modeled responses and determined that a threshold would be the point at which there was an apparent change in behavior. The lowest dose of allergen required to elicit an eosinophilic response is 2 μg, whereas that required to induce an IgG_1 response is 0.5 μg. (Figure 5E). Of interest, these doses elicit responses that are approximately 20% of the maximal

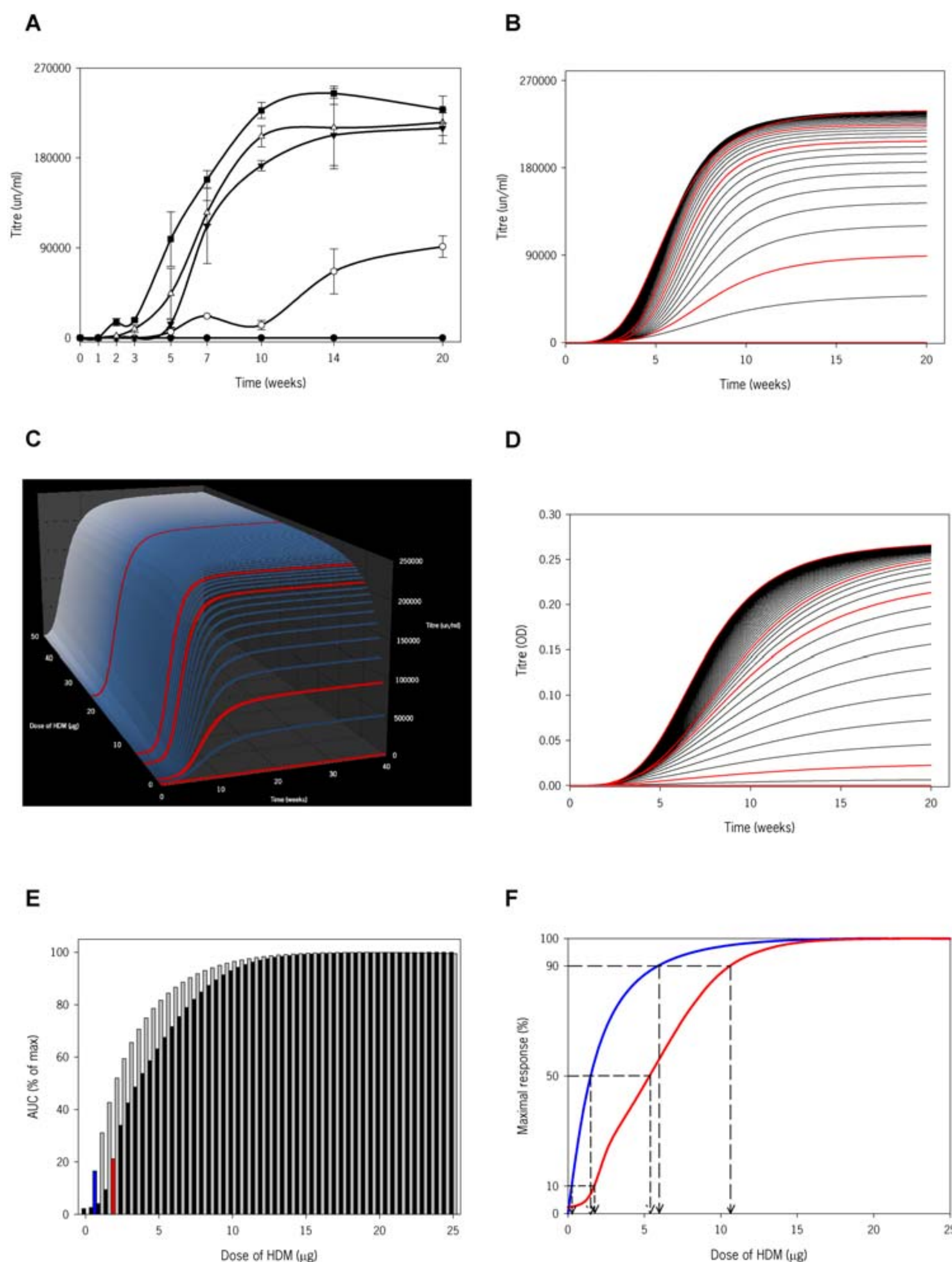
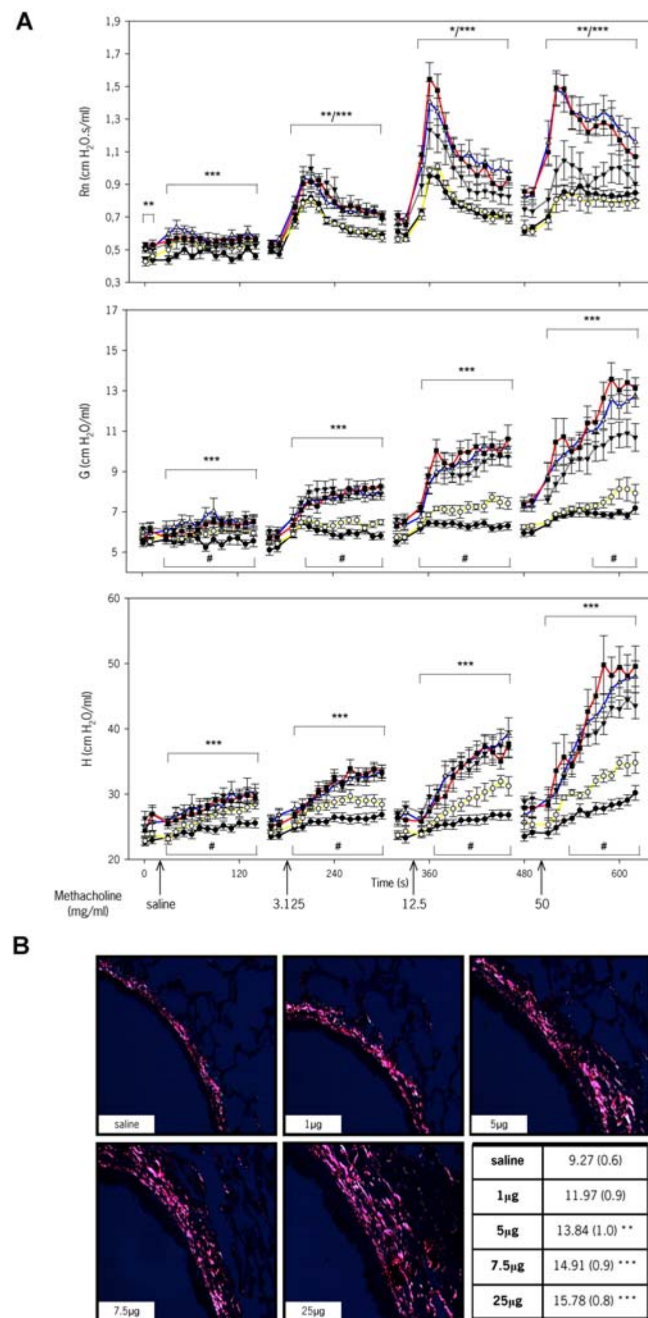


Figure 5. Systemic responses in HDM-exposed mice, subsequent mathematical modeling and comparison between inflammation and sensitization. In panels **B**, **C** and **D**, the simulations for the doses of HDM used experimentally are highlighted in red. (**A**) Serum levels of HDM-specific IgG_1. BALB/C mice were exposed to either saline (solid circles) or HDM, 1 µg (open circles), 5 µg (solid triangles down), 7.5 µg (open triangles up) or 25 µg (solid squares) for up to 20 weeks. Data represent mean±s.e.m. (n = 2–9 mice/group). (**B**) Mathematical model for HDM-specific IgG_1. A simple mathematical model was developed and IgG_1 levels over time and at doses ranging from 0 to 25 µg of HDM, in 0.5 µg increments, were predicted. (**C**) 3D representation of HDM-specific IgG_1 responses, including predictions up to 50 µg and 40 weeks of HDM exposure. (**D**) Mathematical model for HDM-specific IgE. A simple mathematical model based on serum measurements was developed and IgE levels were simulated over time and at doses ranging from 0 to 25 µg of HDM in 0.5 µg increments. (**E**) Area under the curve (AUC) of the maximal number of eosinophils (black bars) and level of HDM-specific IgG_1 (grey bars). The lower dose showing a change in the behavior of the curve (threshold dose), is identified for HDM-specific IgG_1 (blue bar, 0.5 µg) and eosinophilia (red bar, 2 µg). The results are based on computer simulations. (**F**) Maximal responses for HDM-specific IgG_1 (blue line) and eosinophilia (red line) at a range of doses of HDM. The 90% of the maximal inflammatory or immunoglobulin response (long dashed line) is reached when given about 11 or 6 µg of HDM, respectively; approximately 2 and 5 µg of HDM are required to elicit 50% of the maximal inflammatory and immunoglobulin responses (medium dashed line), and <1 and 2 µg to induce 10% of these responses (short dashed line).
doi:10.1371/journal.pone.0002426.g005

inducible response. It is clear that the pattern of the areas under each curve for IgG_1 and eosinophils are similar; however, the latter is shifted to the right indicating that the amount of allergen required to elicit not only the lowest response but all responses is different. To better visualize this, and to standardize measurements, we plotted each outcome as a percentage of the maximal response. As shown in Figure 5F, any level of sensitization is achieved with about half the amount of allergen required to



saline	9.27 (0.6)
1µg	11.97 (0.9)
5µg	13.84 (1.0) **
7.5µg	14.91 (0.9) ***
25µg	15.78 (0.8) ***

Figure 6. Physiological and structural lung changes in mice exposed to different doses of HDM. (**A**) Analysis of airway responsiveness to methacholine (MCh) in mice exposed to HDM for 3 weeks. Airway resistance (Rn), tissue dampening (G) and tissue elastance (H) were determined in BALB/C mice exposed to either saline (solid circles, black line) or HDM, 1 µg (open circles, yellow line), 5 µg (solid triangles down, grey line), 7.5 µg (open triangles up, blue line) or 25 µg (solid squares, red line). A time-course of 2 baseline measurements prior to nebulization of increasing doses of MCh (0, 3.125, 12.5 and 50 mg/ml) followed by 12 consecutive measurements is shown. Data represent mean±s.e.m. (n = 5–12 mice/group). *, ** and *** indicate $p<0.05$, <0.01 and <0.001, respectively, in mice exposed to 5, 7.5 and 25 µg compared to saline; # indicates $p<0.05$ in mice exposed to 1 µg compared to saline. (**B**) Airway remodeling after 7 weeks of HDM exposure. Picro Sirius red-stained lung sections visualized under polarized light (×20) and morphometric analysis show increased subepithelial accumulation of collagen in HDM exposed mice. Data represent the mean of the percentages of stained area of interest (±s.e.m.). ** indicates $p<0.01$ and ***, $p<0.001$ versus saline exposed mice (n = 7–12 mice/group).
doi:10.1371/journal.pone.0002426.g006

achieve the same level of eosinophilic inflammation. Higher IgG_1 responses are not only induced by the same amount of allergen but also greater changes in IgG_1 are observed at lower doses of allergen. Similar observations were made for TCN and IgE (data not shown).

Lung function and remodeling

Airway dysfunction, notably airway hyperreactivity (AHR), is a hallmark of allergic asthma. Preliminarily, we have evaluated lung function to a range of doses after 3 weeks of HDM exposure, a time-point where there is prominent inflammation but no airway remodeling [15]. As shown in Figure 6A, airway resistance (Rn), tissue dampening (G) and elastance (H) increase dose-dependently, being severe in mice exposed to 7.5 and 25 µg of HDM. Most of the peripheral effects observed at these doses of allergen exposure, as measured by G and H, can be explained as airway closure with some elements of lung heterogeneity, as assessed by hysteresivity (η, data not shown) [19]. Rn in mice exposed to 1 µg of HDM was not significantly different than that in saline-treated animals. Interestingly, G and H seemed to be increased in these mice suggesting incipient functional abnormalities occurring prior to detectable inflammation. To note, mice exposed to 5, 7.5 and 25 µg of HDM had a significantly higher baseline Rn compared to the 1 µg and saline groups, indicating a degree of permanent narrowing of the conducting airways. A comprehensive clarification of the variables that influence airway function in this system will require not only the acquisition of an extensive set of functional data but also of additional data including mucous production, permeability and airway structural changes, i.e. remodeling. In specific regard to the later, Figure 6B shows that subepithelial collagen deposition increases in a dose-dependent manner after 7 weeks of allergen exposure; changes in mice exposed to 1 µg of HDM were not significant compared to saline. Clearly, a quantitative delineation of the relationships between tissue and functional variables with inflammatory and immune variables is a major computational challenge beyond the scope of the research presented here.

Discussion

Understanding immune responsiveness will benefit from accepting the multidimensionality and quantitative nature of immunological phenomena [20]. Here, we have engaged this precept to investigate immune-inflammatory responses following repeated HDM exposure in mice. The computational analysis we have performed allows for the identification of rules and parameters that define the system. Principal rules are that relationships between time and infiltrating total cells, as well as mononuclear cells and neutrophils, and serum immunoglobulins follow a logistic-like curve; in sharp contrast, the eosinophil response over time follows a bell shaped-like curve. These rules presuppose a dynamic behavior with at least one significant implication: the lung cellular effector profile quite drastically changes depending on dose and length of exposure to allergen. These multiple possible outcomes may be mathematically viewed as a demonstration of heterogeneity.

The distinct behavior of eosinophils is intriguing. The underlying immunological explanation is unknown at this time; however, it seems intuitive that if allergen exposure is considered as an input, persistent deliverance of such an input will stress the

system and instigate reactive responses. From this perspective, the decrease of eosinophils and the increase in mononuclear cells are likely to be mechanistically related. Flow cytometric analysis delineating the changes in the dynamics of subsets of mononuclear cells (T cells and monocyte/macrophages) over the entire protocol will be informative and suggest future venues of research.

Several parameters define the behavior of the system. First, we have identified a threshold dose at approximately 0.5 μg of HDM for sensitization and 2 μg for inflammation. In fact, exposure to 2.5 μg of HDM elicits a detectable eosinophilic inflammatory response (data not shown). Second, responsiveness for all constituents is dependent on the strength of the initial dose of allergen; moreover, the greater the input, the steeper the initial slope of the response. Third, the system has an inherently limited capacity to respond, at least to the same allergen. This maximal responsiveness is achieved at a dose between 10 and 15 μg, and further increases in dose or length of exposure do not result in greater responses. Fourth, there is an entire range of responses between the threshold and the maximum; mathematically, however, the model reveals that the distribution of responses is non-linear. Lastly, a comparative analysis of inflammation and sensitization outputs reveals that the development of the latter is more sensitive to allergen than the induction of airway inflammation. That the relationships between exposure and either sensitization or inflammation are non-linear intimates that the relationship between sensitization and inflammation is non-linear as well. It is tempting to speculate that these findings may contribute to explain the difference between the prevalence of atopy (~40%) and asthma (5–10%) in humans [21,22,23,24].

The question of how the concentrations of allergen used here compare to human exposure is elusive because the terms of reference are precarious (reviewed in [25]). Many studies have examined the amount of mite allergen present in homes. However, the numbers vary extraordinarily. Not only is there a plethora of environmental variables influencing the concentration of mite allergens in the household but there are also several collection and measurement techniques [3,26,27,28]. In addition, the relationship between the micrograms of allergen measured in a dust sample and the amount of allergen that is airborne, inspired, and reaches the lower airway is enigmatic. Indeed, the inability to precisely determine mucosal HDM exposure in humans frustrates the justifiable desire to formulate a rigorous interspecies comparison of exposures. Perhaps such a straightforward comparison is an ill-conceived goal; arguably, numbers may not be translated between species but behaviors likely can.

Many issues have not been addressed here. For example, experiments were conducted in BALB/C mice. While we know that C57BL/6 mice respond to HDM even more vigorously in terms of inflammation, it definitely cannot be assumed that the behavior of these two strains, or others, is identical. Similarly, these experiments were performed in female mice and, thus, a direct application to male mice is unadvisable. In addition, we cannot presume that the behaviors described for HDM apply to other aeroallergens. With these limitations, our research furnishes a conceptual foundation and operating tools for the evaluation of other variables or system perturbations of a pharmacological, environmental or genetic nature. Based on the present research, future analysis of immune responses exploring these variables may not require the generation of entire data sets but of selected experiments to generate comparative algorithms to re-define the overall behavior of the system.

There has been a considerable interest by engineers, mathematicians and computer scientists in the application of their skills to modeling biological processes. Over the last few years, biologists have shown an increasing attraction to join in this enterprise. Arguably, the catalyst underlying this initiative has been the recognition that biological processes are, formally, complex processes. As such, efforts to incorporate new conceptual and experimental stratagems must be made to better comprehend them. The development of mathematical modeling based upon research, primarily *in vitro*, examining hemopoiesis and stem cell renewal [29], models of virus-immune dynamics [30] and cancer cell propagation [31] typify these efforts. Particularly in the area of inflammation, agent-based and equation-based models have been established to provide insight into the complex dynamics of this process [32,33,34,35,36,37]. However, the research presented in this manuscript is, to our knowledge, the first to investigate the interaction between aeroallergens and the immune system *in vivo* from a computational perspective.

Supporting Information

Figure S1 Iterations to validate the mathematical model for the inflammatory response. (A) A mathematical equation was developed based on the responses to saline, 1, 7.5 and 25 μg of HDM up to 14 weeks (blue lines). Simulations (red lines) for these doses studied were generated. Then, the equation was used to predict the response to 5 ug, which was subsequently evaluated experimentally (blue line, triangles down). (B) A refined mathematical equation was developed based on the responses to saline, 1, 5, 7.5 and 25 ug of HDM up to 14 weeks (blue lines). Simulations (red lines) for these doses were generated. Then, responses for all doses at 20 weeks were predicted, and these were subsequently evaluated experimentally.
Found at: doi:10.1371/journal.pone.0002426.s001 (1.50 MB TIF)

Methods S1 Supplementary methods, including elaboration of mathematical Equations 1 to 6.4 used in the models, validation analysis and area under curves.
Found at: doi:10.1371/journal.pone.0002426.s002 (0.11 MB PDF)

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Author Contributions

Conceived and designed the experiments: MJ AL MC. Performed the experiments: AL MC DC CM NS TW. Analyzed the data: MJ AL MC LL ML. Wrote the paper: MJ AL MC. Other: Contributed writing the paper: AC ML PO LL. Analyzed biological samples: SG AL.

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Methods S1 (Supporting Information)

Mathematical and computational modeling

Outcomes were analyzed by individual doses over specific time periods, deriving a general equation for all doses and all times. Within each equation, numerical coefficients were analyzed based on dose, establishing dose-dependent equations. In some cases, equations were subsequently re-adjusted manually. In order to capture all of the characteristics for a given outcome, *complex* models were derived based on the dynamics of the experimental data. In the case of TCN and EOS, equations were sectioned in parts according to time intervals where it was visually apparent that the curvature of the responses changed; the time-points of 3, 5, and 7 weeks were selected for TCN, and EOS was split at 7 weeks. Through the incorporation of data from additional doses and time-points, the model proved to be modular, i.e. the inclusion of new data did not require the derivation of a new equation, but rather only re-adjustment of the existing model. After performing simulations with the *complex* models, it was determined that *simple* models would yield the general characteristics of each outcome with more clarity and conciseness. Such *simple* models were generated for TCN, EOS, IgG_1 and IgE using a single equation for all time-points, as opposed to defining dose-responses piecewise. 2D comparison of actual data and simulated data was visualized in SigmaPlot software (Systat Software Inc.), while 3D simulation images were generated using Microsoft Excel 2007.

The following sets of equations describe the dynamics of each measured outcome (y) in the context of dose (x, in μg of HDM) and time (t, in weeks).

TCN (Complex)

$$y = f_C^{TCN}(x,t) = \begin{cases} x_{a1} + x_{b1} sin(z_1(x,t))^2 & , \quad 0 \le t \le 3 \\ x_{a2} + x_{b2} e^{z_2(x,t)} + x_{g2} t + x_{h2} t^2 & , \quad 3 \le t \le 7 \\ x_{a3} + x_{b3} e^{z_3(x,t)} + x_{g3} e^{z_4(x,t)} & , \quad t \ge 7 \end{cases} \tag{1}$$

where,

$$z_1 = \frac{\pi(t - x_{c1})}{x_{d1}} \tag{1.1}$$

$$z_2 = -0.5\left(\frac{t - x_{c2}}{x_{d2}}\right)^2 \tag{1.2}$$

$$z_3 = -0.5\left(\frac{t - x_{c3}}{x_{d3}}\right)^2 \tag{1.3}$$

$$z_4 = -\left(\frac{t - x_{h2}}{x_{k2}}\right) \tag{1.4}$$

$$x_{a1} = 1.172 - 0.516e^{-0.5\left(\frac{x-12.955}{12.284}\right)^2} \tag{1.5}$$

$$x_{b1} = -0.245 + \frac{4.171}{\left(1 + 10^{-0.103(x+0.252)}\right)^{3.802}} \tag{1.6}$$

$$x_{c1} = 0.029 + 0.377\sin\left(\pi\left(\frac{x - 0.066}{24.889}\right)\right)^2 \tag{1.7}$$

$$x_{d1} = \frac{3.361 + 0.289x + 0.004x^2 - 0.178x^3}{1 + 0.280x + 0.0002x^2 - 0.037x^3} \tag{1.8}$$

$$x_{a2} = \begin{cases} 0.996 - 0.056x + 0.049\left(0.377^x\right) & , \quad 0 \le x \le 5 \\ 5.338\left(1 - e^{-\left(0.065(x-4.382)^{0.606}\right)}\right) & , \quad x > 5 \end{cases} \tag{1.9}$$

$$x_{b2} = \begin{cases} -0.055 + 0.229x - 0.007x^2 & , \quad 0 \le x \le 5 \\ -0.039 + \dfrac{0.958}{1 + \left(\frac{x-5.050}{10.092}\right)^{0.167}} & , \quad x > 5 \end{cases} \tag{1.10}$$

$$x_{c2} = \begin{cases} \dfrac{x+0.157}{0.006+0.220(x+0.157)-0.005(x+0.157)^2} & , \quad 0 \le x \le 5 \\ 5.208-\dfrac{9.888}{x}+\dfrac{47.078}{x^2} & , \quad x > 5 \end{cases} \tag{1.11}$$

$$x_{d2} = \begin{cases} 1.166+\dfrac{0.948}{1+\left(\frac{x}{2.152}\right)^{0.438}} & , \quad 0 \le x \le 5 \\ \dfrac{x-4.562}{0.103+0.401(x-4.562)+0.013(x-4.562)^2} & , \quad x > 5 \end{cases} \tag{1.12}$$

$$x_{g2} = \begin{cases} 0.014+0.002x+0.0001x^2 & , \quad 0 \le x \le 5 \\ 0.149-\dfrac{3.067}{x}+\dfrac{12.508}{x^2} & , \quad x > 5 \end{cases} \tag{1.13}$$

$$x_{h2} = \begin{cases} -0.007+0.004x+0.0006x^2 & , \quad 0 \le x \le 5 \\ 0.039+0.002x^{0.524}-0.0004x^{1.478} & , \quad x > 5 \end{cases} \tag{1.14}$$

$$x_{a3} = \begin{cases} 0.298+0.657x-0.020x^2 & , \quad 0 \le x \le 5 \\ \dfrac{x-4.749}{-0.024+0.423(x-4.749)-0.008(x-4.749)^2} & , \quad x > 5 \end{cases} \tag{1.15}$$

$$x_{b3} = \begin{cases} -0.455-0.216\sqrt{x}+0.347x & , \quad 0 \le x \le 5 \\ -5.056+6.237e^{\frac{-(\ln(x)-2.075)^2}{3.487}} & , \quad x > 5 \end{cases} \tag{1.16}$$

$$x_{c3} = \begin{cases} \dfrac{9.212+5.586x}{1+0.431x-0.013x^2} & , \quad 0 \le x \le 5 \\ 3.101+10.475e^{\frac{-(\ln(x)-2.033)^2}{3.914}} & , \quad x > 5 \end{cases} \tag{1.17}$$

$$x_{d3} = \begin{cases} 4.064+1.785e^{\frac{-x}{1.797}}-2.582e^{\frac{-x}{5.497}}-1.547e^{\frac{-x}{6.004}} & , \quad 0 \le x \le 5 \\ \dfrac{x-4.505}{-0.194+0.808(x-4.505)-0.016(x-4.505)^2} & , \quad x > 5 \end{cases} \tag{1.18}$$

$$x_{g3} = \begin{cases} 0.019 - 0.066x + 0.739\left(0.246^{x}\right) & , \quad 0 \le x \le 5 \\ -0.316 + 6.708e^{\frac{-(x-5.078)}{9.779}}\left(1 - e^{\left(\frac{-(x-5.078)}{13.719}\right)^{0.489}}\right) & , \quad x > 5 \end{cases} \tag{1.19}$$

$$x_{h3} = \begin{cases} 4.527 - 1.986e^{\frac{-x}{0.213}} + 3.123e^{\frac{-x}{9.057}} & , \quad 0 \le x \le 5 \\ 1.127\left(\frac{x}{-3.863 + x}\right) + 3.257\left(\frac{x}{28.574 + x}\right) + 0.178x & , \quad x > 5 \end{cases} \tag{1.20}$$

$$x_{k3} = \begin{cases} 5.291 + 30.559e^{-3.157x} & , \quad 0 \le x \le 5 \\ 7.844 + \frac{-2.847}{1 + \left(\frac{x-4.997}{9.340}\right)^{0.269}} & , \quad x > 5 \end{cases} \tag{1.21}$$

TCN (Simple)

$$y = f_S^{TCN}(x,t) = \frac{x_a + x_b t + x_c t^2}{1 + x_d t + x_g t^2} \tag{2}$$

where,

$$x_a = 0.989 + 0.046\left(\frac{0.665}{1 + 10^{0.407(x-6.214)}}\right) + 0.045\left(\frac{1 - 0.665}{1 + 10^{-0.097(x-18.773)}}\right) - 1.4 \tag{2.1}$$

$$x_b = 0.187 + 1.091\left(\frac{0.978}{1 + 10^{-0.571(x-6.389)}}\right) + 1.091\left(\frac{1 - 0.978}{1 + 10^{-0.081(x-18.780)}}\right) \tag{2.2}$$

$$x_c = 0.378 + 1.137e^{-0.5\left(\frac{x-7.548}{0.595}\right)^2} - 0.239e^{-\left(\frac{x-0.876}{12.035}\right)} \tag{2.3}$$

$$x_d = 0.009 + 1.995\left(\frac{1}{1 + e^{-\frac{x-0.638}{0.273}}}\right)\left(1 - \frac{1}{1 + e^{-\frac{x-0.638}{41.471}}}\right) \tag{2.4}$$

$$x_g = 0.142 + 0.473e^{-0.5\left(\frac{x-7.155}{1.063}\right)^2} + 0.050e^{-\left(\frac{x-0.326}{12.839}\right)} \tag{2.5}$$

EOS (Complex)

$$y = f_C^{EOS}(x,t) = \begin{cases} x_{a1} + x_{b1} e^{z_1(x,t)} + x_{g1} e^{z_2(x,t)} & , \quad 0 \le t \le 7 \\ x_{a2} + x_{b2} t + x_{c2} t^2 + x_{d2} t^3 & , \quad t \ge 7 \end{cases} \tag{3}$$

where,

$$z_1 = -0.5\left(\frac{t - x_{c1}}{x_{d1}}\right)^2 \tag{3.1}$$

$$z_2 = -0.5\left(\frac{t - x_{h1}}{x_{k1}}\right)^2 \tag{3.2}$$

$$x_{a1} = \frac{-0.018 + 0.106x - 0.145x^2 - 0.005x^3}{1 + 0.096x - 0.151x^2 - 0.005x^3} \tag{3.3}$$

$$x_{b1} = \frac{0.057x^{1.633}}{1 + 0.036x^{1.633}} \tag{3.4}$$

$$x_{c1} = 2.595 - 2.021e^{\frac{-x}{10.062}} + 2.935e^{\frac{-x}{32.085}} \tag{3.5}$$

$$x_{d1} = \frac{7.088 + 0.903x - 0.199x^2}{1 + 0.048x - 0.113x^2} \tag{3.6}$$

$$x_{g1} = \frac{0.045x^{2.665}}{1 + 0.031x^{2.665}} \tag{3.7}$$

$$x_{h1} = 10.578 + 0.234x - 0.019x^2 + 0.0004x^3 \tag{3.8}$$

$$x_{k1} = 7.078 - 0.097\sqrt{x} + 0.076x \tag{3.9}$$

$$x_{a2} = -0.233\left(\frac{x}{0.553+x}\right) + 0.508\left(\frac{x}{4.993+x}\right) + 0.176x \tag{3.10}$$

$$x_{b2} = \begin{cases} -0.066 + \dfrac{0.110}{1+10^{0.232x-0.109}} & , \quad 0 \le x \le 5 \\ 0.335 - 0.019x & , \quad x > 5 \end{cases} \tag{3.11}$$

$$x_{c2} = \frac{x}{512.820 + 205.158x + 324.354\sqrt{x}} \tag{3.12}$$

$$x_{d2} = 2.590E^{-06} - 1.509E^{-05}x + 5.980E^{-07}x^2 \tag{3.13}$$

EOS (Simple)

$$y = f_S^{EOS}(x,t) = \left| \frac{t + x_a}{x_b + x_c(t + x_a) + x_d(t + x_a)^2} \right| \tag{4}$$

where,

$$x_a = -0.047 - 0.019\ln(x + 0.081) \tag{4.1}$$

$$x_b = 3.737 + 46.203e^{\frac{-(x-0.180)}{2.148}} \left| 1 - e^{\frac{-(x-0.180)}{12.861}} \right|^{0.145} \tag{4.2}$$

$$x_c = -0.615 + 5.029e^{-0.987x} \tag{4.3}$$

$$x_d = 0.200 + \frac{7.909}{1+10^{1.439x-1.389}} \tag{4.4}$$

IgE (Simple)

$$y = f_S^{IgE}(x,t) = \frac{x_a t^{x_d}}{x_c^{\,x_d} + t^{x_d}} \tag{5}$$

where,

$$x_a = 0.272\left(1 - e^{\left(-0.297(x-0.015)\right)^{1.819}}\right) \tag{5.1}$$

$$x_c = 6.737 + 70.018\left(\frac{20.601}{4(x-1.682)^2 + 20.601^2}\right) \tag{5.2}$$

$$x_d = \frac{17.942 + 9.965x^{1.562}}{9.111 + x^{1.562}} \tag{5.3}$$

IgG₁ (Simple)

$$y = f_S^{IgG_1}(x,t) = x_a + \frac{x_b - x_a}{1 + \left(\frac{t}{x_c}\right)^{x_d}} \tag{6}$$

where,

$$x_a = \frac{x}{1.06E^{-05} + 4.583E^{-06}x - 4.237E^{-06}\sqrt{x}} \tag{6.1}$$

$$x_b = \frac{-244.738x}{1.527 + x} \tag{6.2}$$

$$x_c = 5.752 + \frac{4.489}{1 + 10^{0.855(x-1.261)^{0.182}}} \tag{6.3}$$

$$x_d = 2.133 + \frac{7.243x}{1.915 + x} + \frac{-8.133x}{16.102 + x} \tag{6.4}$$

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*Note: All numerical coefficients above are given to 3 decimal places for brevity, however all equations were computed using the full coefficients as derived by the model.

Validation analysis

Linear regression analysis and 95% CI were used to validate the mathematical models since they are deterministic and non-deterministic approaches, respectively [1]. *Deterministic validation metric:* Actual data was plotted against model data and linear regression performed with the condition that the regression line must pass through (0,0). The possible goodness of fit statistic, R^2 (coefficient of determination), was calculated. An R^2 of 1 would mean that all values of actual data and model data are equal and thus lie upon the line y=x. *Non-deterministic validation metric:* 95% CIs were created using the appropriate t probability distribution ($t_{0.05,\, df}$), depending on the number of degrees of freedom (number of mice sacrificed minus 1) at each time (t) and dose (x) with an α of 0.05. In addition, as proposed by Oberkampf, *et al.* [2], global validation metrics to quantify overall model accuracy were calculated as follows:

$$\text{Average Relative Accuracy of Model } = 1 - \left[\frac{1}{x_{\max}} \int_0^{25} \frac{1}{t_{final}} \int_0^{t_{final}} \left| \frac{\overline{y_a} - y_m}{\overline{y_a}} \right| dtdx \right]$$

where y_a is the actual mean (obtained from experiment), y_m is the predicted mean (virtual data). Definite integrals, estimated by trapezoidal Riemann sum, are used instead of straight summation in order to be able to assess predictive (interpolation) capabilities of each model. A similar method is applied to calculate the confidence indicator (half-width 95% CIs are averaged over both time and dose):

$$\text{Confidence Indicator} = \left[\frac{1}{x_{\max}} \int_0^{x_{\max}} \frac{1}{t_{final}} \int_0^{t_{final}} \frac{SD}{\sqrt{n_{t,x}}} \frac{t_{0.05,df}}{\overline{y_a}} dtdx \right]$$

The average relative accuracy of the model, in line with assessment using linear regression, respectively calculated our initial, second and final *complex* TCN models to be 94.23±21.55%, 95.49±22.67% and 97.50±22.42% accurate (to a maximum of a 100%) with 95% confidence. Comparing between *complex* and *simple* TCN models, the *complex* TCN model again provides slightly more accurate predictions than the *simple* model, 97.50±22.42% versus 89.03±22.42% accuracy with 95% confidence. Further, our models (both *simple* and *complex*) fall within the 95% CI band meaning that there is a high

probability for our model to be able to predict real responses. Comprehensive non-deterministic analysis supports our findings from linear regression and allows for inferences to be made about our models' predictive capabilities.

Area under curves

For each outcome (IgG_1 and EOS), equations that represented each increment of 0.5μg HDM, dose-response curves were exported into Wolfram Mathematica software (Wolfram Research Inc.) to calculate the definite integral from 0 to 20 weeks of each equation.

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Chapter 3.

IL-33, but not Thymic Stromal Lymphopoietin or IL-25, is Central to Mite and Peanut Allergic Sensitization

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Mechanisms of allergy and clinical immunology

IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization

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Background: Allergen exposure at lung and gut mucosae can lead to aberrant T_H2 immunity and allergic disease. The epithelium-associated cytokines thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 are suggested to be important for the initiation of these responses.
Objective: We sought to investigate the contributions of TSLP, IL-25, and IL-33 in the development of allergic disease to the common allergens house dust mite (HDM) or peanut.
Methods: Neutralizing antibodies or mice deficient in TSLP, IL-25, or IL-33 signaling were exposed to HDM intranasally or peanut intragastrically, and immune inflammatory and physiologic responses were evaluated. *In vitro* assays were performed to examine specific dendritic cell (DC) functions.
Results: We showed that experimental HDM-induced allergic asthma and food allergy and anaphylaxis to peanut were associated with TSLP production but developed independently of TSLP, likely because these allergens functionally mimicked TSLP inhibition of IL-12 production and induction of OX40 ligand (OX40L) on DCs. Blockade of OX40L significantly lessened allergic responses to HDM or peanut. Although IL-25 and IL-33 induced OX40L on DCs *in vitro*, only IL-33 signaling was necessary for intact allergic immunity, likely because of its superior ability to induce DC OX40L and expand innate lymphoid cells *in vivo*.
Conclusion: These data identify a nonredundant, IL-33–driven mechanism initiating T_H2 responses to the clinically relevant allergens HDM and peanut. Our findings, along with those in infectious and transgenic/surrogate allergen systems, favor a paradigm whereby multiple molecular pathways can initiate T_H2 immunity, which has implications for the conceptualization and manipulation of these responses in health and disease. (J Allergy Clin Immunol 2013;131:187-200.)

Key words: *T_H2, allergy, asthma, house dust mite, peanut, thymic stromal lymphopoietin, IL-25, IL-33, OX40 ligand, innate lymphoid cells*

Allergies are immune-mediated hypersensitivity diseases mainly driven by T_H2 responses to normally tolerated environmental antigens. Allergic asthma is a chronic lung disease characterized by airway infiltration of T_H2 cells and eosinophils, increased mucus production, and reversible airway obstruction. It is estimated that 10% to 20% of the population is allergic to house dust mite (HDM), the most ubiquitous and pervasive aeroallergen worldwide.[1,2] Food allergy causes symptoms that range from mild erythema and pruritus to acute, life-threatening systemic reactions termed anaphylaxis. Peanut allergy affects approximately 1% to 2% of the general population[3,4] and is highly associated with anaphylaxis.[5]

Despite substantial advances in understanding the pathophysiology of these diseases, the initial cellular and molecular events that cause susceptible subjects to acquire allergic disease are not well understood. Research on the origin of allergic disease in human subjects is exceedingly difficult because sensitization is often clinically silent. Thus the immunologic basis of allergic sensitization is largely investigated in animal models.

Mechanistically, allergic responses have been proposed to be initiated after epithelial cell contact with antigens that leads to the production of a number of cytokines able to promote T_H2 immunity.[6] Paramount among these has been thymic stromal lymphopoietin (TSLP), which has been reported to condition dendritic cells (DCs) to favor T_H2 induction through 2 main actions[7]: (1) limitation of IL-12 production[8-10] and (2) upregulation of the costimulatory molecule OX40 ligand (OX40L).[11,12] Since the identification of TSLP, additional epithelium-associated cytokines also capable of inducing T_H2 immunity have been discovered, notably IL-25 (IL-17E), which signals through IL-17 receptor B

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Abbreviations used
- BMDC: Bone marrow–derived dendritic cell
- CT: Cholera toxin
- DC: Dendritic cell
- HDM: House dust mite
- IL-17RB: IL-17 receptor B
- ILC: Innate lymphoid cell
- KO: Knockout
- LN: Lymph node
- OVA: Ovalbumin
- OX40L: OX40 ligand
- STAT6: Signal transducer and activator of transcription 6
- TSLP: Thymic stromal lymphopoietin
- TSLPR: Thymic stromal lymphopoietin receptor
- WT: Wild-type
- VSV: Vesicular stomatitis virus

(IL-17RB),[13,14] and IL-33, which signals through the ST2 receptor.[15] A majority of studies supporting a role for TSLP, IL-25, and/or IL-33 in the generation of allergic responses in the lung and gut have relied on conventional models of nonmucosal intraperitoneal sensitization to the surrogate allergen ovalbumin (OVA) adsorbed to aluminum-based adjuvants, such as aluminum hydroxide (alum). Thus how these cytokines initiate T_H2 sensitization to common allergens at mucosal sites remains to be elucidated.[16] Recent reports showed that intraperitoneal injection of recombinant IL-25, IL-33, or both can expand novel innate lymphoid cell (ILC) populations in the lung and gut that are able to produce T_H2 cytokines and promote host defense[17]; however, the presence and contribution of these cells during allergic sensitization remain unknown.

Here we sought to better understand how TSLP, IL-25, and IL-33 influence sensitization to the common allergens HDM and peanut in the lung and gut, respectively. To accomplish this, we used established models of HDM-induced allergic asthma[18,19] and peanut allergy and anaphylaxis.[20,21] In stark contrast to the increasingly prevalent opinion that TSLP is a master regulator of T_H2 responses and thus atopic disease,[22] we found that TSLP was dispensable for the generation of IL-4–dependent humoral and cellular immunity to HDM or peanut. Similar results were observed with respect to IL-25. In contrast, IL-33 played an essential role. In fact, our findings delineate an IL-33/OX40L/ILC pathway leading to T_H2 immunity in response to 2 clinically relevant allergens at 2 different mucosal sites.

METHODS

Supplemental information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Mice

Adult (6-8 weeks old) female BALB/c and C57BL/6 mice, thymic stromal lymphopoietin receptor (TSLPR) knockout (KO; B6 or C.129P2-*Crlf2*^{tm1Jni}) and IL-17RB KO (B6.129-*Il17rb*^{tm1Bud}; gifts from Amgen, Thousand Oaks, Calif) mice and ST2 KO mice (C.129P2-*Il1rl1*^{tm1Anjm}; gift from MedImmune, Gaithersburg, Md), were obtained from Charles River (San Diego, Wilmington, Saint-Constant, Quebec, Canada). IL-4 KO (B6.129P2-*Il4*^{tm1Cgn}) and wild-type (WT) control animals were from the Jackson Laboratory (Bar Harbor, Me). IL-13 KO (C.129P2-*Il13*^{tm2Anjm}) and signal transducer and activator of transcription 6 (STAT6) KO (B6.129S-*Stat6*^{tm1Jni}) mice were bred in house. All experiments were performed with age-, sex-, and strain-matched control animals and were approved by the Animal Research Ethics Board of McMaster University (Hamilton, Ontario, Canada).

Statistics

Data were analyzed and graphed with GraphPad Prism 5 software (GraphPad Software, San Diego, Calif). Continuous data are expressed as means ± SEMs and were analyzed by using the *t* test (unpaired, 2-tailed) or 1- or 2-way ANOVA with Dunnet or Bonferroni *post hoc* tests. Temperature data were analyzed with repeated measures. Ordinal data are shown as individual mice with medians and were analyzed with Mann-Whitney *U* tests. Differences were considered statistically significant at a *P* value of less than .05.

RESULTS

Airway sensitization to HDM does not require TSLP signaling

We sought to investigate the requirement of TSLP in experimental allergic asthma to the clinically relevant allergen HDM. This model[23] solely involves mucosal exposure, does not use any exogenous adjuvant, and critically requires the canonical T_H2-inducing molecule IL-4.[24] Consistent with a previous report,[25] TSLP was detected in the lungs of naive mice, and levels were increased upon 3-day HDM exposure (Fig 1, *A*), a protocol that induces sensitization without overt inflammation. TSLP upregulation was not detected in sera (data not shown), suggesting that cytokine signaling took place locally. Thus allergic sensitization to HDM is associated with TSLP production in the lung.

To test the functional role of TSLP signaling during sensitization, we used a 10-day intranasal HDM exposure protocol that recapitulates early features of the allergic diathesis.[18,19] TSLPR KO mice produced levels of T_H2-associated immunoglobulins (IgG_1 and IgE) comparable with those in WT HDM-exposed mice (Fig 1, *B* and *C*). Accordingly, the proportion of activated $CD69^+$ B cells was similar in both groups (Fig 1, *D*). Likewise, overall airway inflammation was comparable in HDM-exposed WT and TSLPR KO mice (Fig 1, *E*), with a similar degree of eosinophil influx (Fig 1, *F*). Histopathologic evaluation of lung sections showed comparable peribronchial and perivascular inflammatory responses and bronchial goblet cell hyperplasia/hypertrophy with mucus production (Fig 1, *G* and *H*). T-cell activation *in vivo* and *ex vivo* cytokine production were intact regardless of the presence or absence of TSLPR (Fig 1, *I* and *J*).

Similar results were observed in BALB/c mice treated before and throughout the HDM exposure protocol with a neutralizing anti-TSLP antibody (see Fig E1, *A-C*, in this article's Online Repository at www.jacionline.org), as well as in C57BL/6 TSLPR KO mice (see Fig E1, *D-F*). Collectively, these data show that allergic sensitization and airway inflammation in response to HDM occur independently of TSLP and its receptor.

These data, generated by using the common aeroallergen HDM, are in sharp contrast to the results published by 2 different groups reporting TSLP-dependent lung T_H2 responses to the surrogate allergen OVA in mouse models involving intraperitoneal alum-driven sensitization protocols.[26,27] This discrepancy was not due to colony differences because we observed similar TSLPR dependency in OVA plus alum–sensitized mice (see Fig E1, *A-H*). Thus although our data confirm a role for TSLP in this OVA model, they do not support the contention that TSLP is pervasively a key factor in the initiation of allergic asthma.

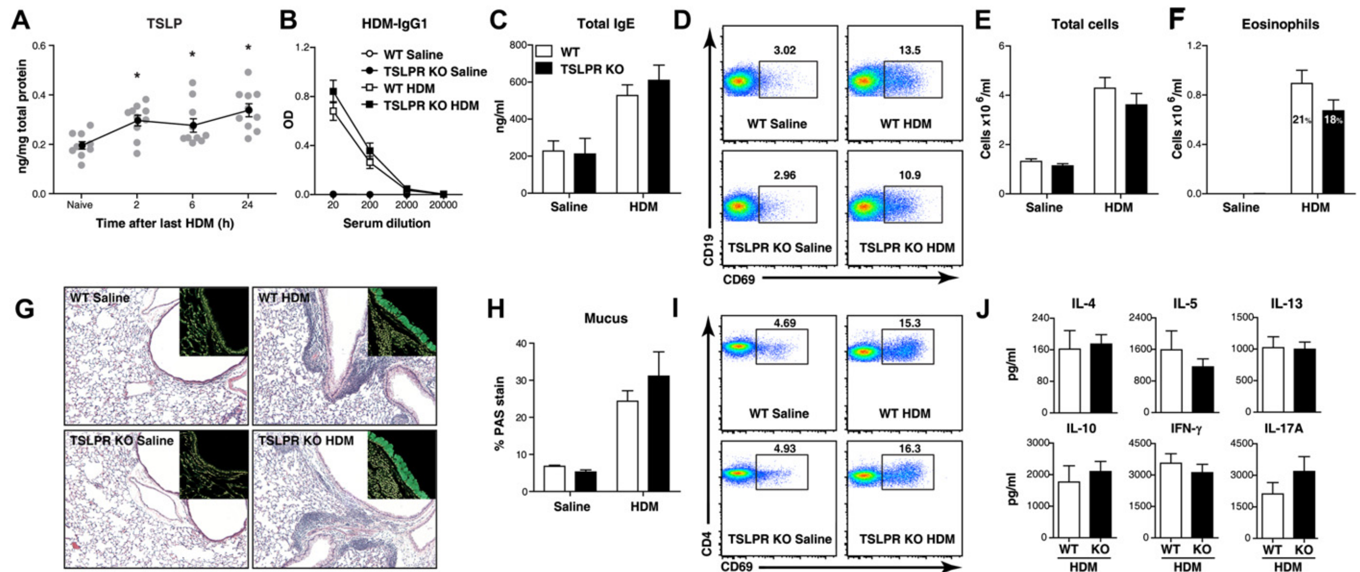

FIG 1. HDM-induced TSLP is not required for allergic sensitization. **A**, TSLP in lung homogenates from naive mice or after 3 days of HDM. **B-J**, BALB/c WT or TSLPR KO mice were exposed to saline or HDM for 10 days. HDM-specific IgG_1 (Fig 1, *B*) and total IgE (Fig 1, *C*) levels in serum. Representative lung $CD45^+CD3^-CD19^+$ B cells expressing CD69 (Fig 1, *D*). Total (Fig 1, *E*) and eosinophil (Fig 1, *F*) airway infiltrates in bronchoalveolar lavage fluid; mean percentage of eosinophils are shown in *bars*. Fig 1, *G*, Representative hematoxylin and eosin–stained lung sections (×100 magnification); *insets* show color-inverted periodic acid–Schiff *(PAS)*–stained sections (×400 magnification). Fig 1, *H*, Morphometric quantification of periodic acid–Schiff staining. Fig 1, *I*, Representative CD69 expression by lung $CD45^+CD3^+CD4^+$ T cells. Fig 1, *J*, Cytokine levels in supernatants of splenocytes stimulated with HDM. Shown are 2 to 3 experiments with 3 to 6 mice per group per experiment. $^*P < .05$ vs naive.

Gastrointestinal sensitization to peanut is IL-4 and STAT6 dependent, but does not require TSLP signaling or basophils

To investigate whether the previous results were idiosyncratic of the site and allergen used, we analyzed the role of TSLP in the induction of T_H2 immunity in the gastrointestinal tract. We have previously shown that intragastric exposure of mice to peanut along with cholera toxin (CT) induces strong gastrointestinal T_H2 responses, such that subsequent peanut challenge elicits systemic anaphylaxis.[20,28] Previously reported mRNA analysis of steady-state intestinal *tslp* expression showed the highest levels in the caecum and large intestine.[10] After ingestion of peanut plus CT for 3 days, we observed enhanced TSLP protein levels in the duodenum but not in other intestinal segments (Fig 2, *A*). TSLP was not detected in intestinal washes or sera (data not shown). Thus the initiation of peanut allergy is associated with TSLP upregulation in the small intestine.

We next attempted to induce gastrointestinal T_H2 immunity to peanut in the absence of TSLP signaling. Both WT and TSLPR KO mice sensitized to peanut produced similarly increased levels of peanut-specific IgG_1 and IgE (Fig 2, *B* and *C*). Functionally, both groups of mice experienced similar anaphylactic responses upon peanut challenge, as measured by comparable decreases in core body temperature, increases in hematocrit levels (a physiologic measure of systemic vascular leakage), and development of clinical signs ranging from pruritus to seizure or death (Fig 2, *D-F*). Using B cell–independent but $CD4^+$ T cell–dependent peritoneal eosinophilic inflammation 72 hours after intraperitoneal challenge as an *in vivo* measure of T-cell function (data not shown),[20] we found robust responses in both the peanut-sensitized and challenged groups (Fig 2, *G* and *H*). Furthermore, *ex vivo* splenocyte cytokine production was also comparable in both groups (Fig 2, *I*).

Anti-TSLP antibody administration throughout sensitization generated similar results (see Fig E2, *A-C*, in this article's Online Repository at www.jacionline.org and data not shown). We also considered that the type of antigen, adjuvant, or route of administration might have affected TSLP dependency, but WT and TSLPR KO mice sensitized with OVA plus CT intragastrically, peanut plus alum intraperitoneally, peanut plus CT intraperitoneally, or the conventional peanut plus CT intragastrically produced similarly increased levels of antigen-specific IgG_1 and IgE and, upon challenge, comparable anaphylactic responses (see Fig E2, *D-O*, and data not shown). Collectively, these results show that intact TSLP signaling is not required for gastrointestinal T_H2 priming and subsequent humoral or cellular food-induced allergic responses.

Basophils, which can respond to and produce TSLP, have been reported to be critical for T_H2 priming in some experimental systems.[29,30] Anti-CD200R3 antibody (clone Ba103) depletion of basophils during HDM exposure was recently reported to partially decrease eosinophilic airway inflammation but not alter T_H2 priming and cytokine production.[31] In the case of peanut, depletion of basophils using anti-asialo GM1[32] or Ba103[21] did not impair peanut-specific IgG_1 or IgE production and subsequent anaphylactic responses to peanut (see Fig E2, *P-R*, and data not shown). Hence basophils are not required for HDM or peanut sensitization.

TSLP has been proposed to be a master regulator of T_H2 immunity and reported to require IL-4.[33,34] Indeed, canonical T_H2 differentiation requires IL-4–mediated activation of STAT6. We have shown that HDM-induced allergic asthma requires IL-4.[24]

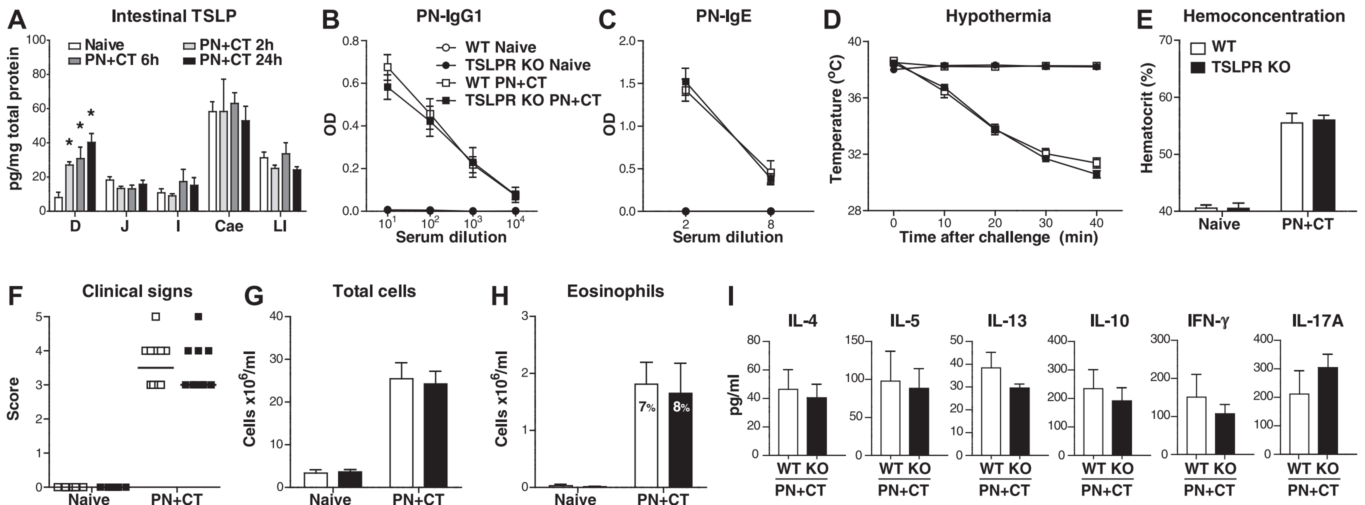

FIG 2. Peanut *(PN)*–induced TSLP is not required for allergic sensitization. **A**, TSLP in tissues from naive mice or after 3 days of peanut CT. *Cae*, Caecum; *D*, duodenum; *I*, ileum; *J*, jejunum; *LI*, large intestine. **B-I**, C57BL/6 WT or TSLPR KO mice were sensitized to peanut and, 1 week later, challenged intraperitoneally. Serum levels of peanut-specific IgG_1 (Fig 2, *B*) and IgE (Fig 2, *C*) 1 day before challenge. Anaphylactic assessment by core body temperature (Fig 2, *D*), hematocrit levels (Fig 2, *E*), and clinical signs (Fig 2, *F*). Total (Fig 2, *G*) and eosinophil (Fig 2, *H*) infiltrates in peritoneal lavage; mean percentages of eosinophils are shown in *bars*. Fig 2, *I*, Cytokine production by peanut-stimulated splenocytes. Shown are 2 to 3 experiments with 3 to 8 mice per group per experiment. $^*P < .05$ versus naive.

We considered that the peanut allergy model did not induce canonical T_H2 induction and hence did not require TSLP. STAT6 or IL-4 KO mice exposed to peanut plus CT did not produce any detectable levels of peanut-specific IgG_1 or IgE (data not shown) or show any clinical or physiologic indication of anaphylaxis upon challenge (see Fig E3, *A-F*, in this article's Online Repository at www.jacionline.org). In contrast, IL-13 KO mice generated robust IgG_1 and anaphylactic responses (see Fig E3, *G-I*). Additionally, peanut-stimulated splenocytes from peanut-sensitized WT and IL-13 KO, but not IL-4 and STAT6 KO, mice produced significantly increased amounts of IL-5 compared with levels seen in naive mice (not shown). Therefore this peanut allergy model is dependent on the canonical T_H2-inducing molecules IL-4 and STAT6, yet functions independently of TSLP.

Overall, data from 2 distinct models of allergic disease lead us to conclude, at variance with the recent proposition,[7,22,35,36] that TSLP does not play a universal role in initiating T_H2 allergic immunity at mucosal surfaces.

HDM and peanut functionally replace TSLP action on DCs

TSLP has been reported to instruct DCs to facilitate T_H2 responses through limiting the production of IL-12p40[8-10] and inducing OX40L.[11,12] To better understand why the development of HDM-induced allergic asthma and peanut allergy did not require TSLP, we investigated whether these allergens could influence DC behavior. To achieve this, bone marrow–derived dendritic cells (BMDCs) were conditioned with complete medium, TSLP, HDM, or peanut and subsequently stimulated with medium or LPS. Few (<5%) DCs conditioned with medium alone spontaneously produced IL-12p40, whereas more than 50% of DCs were IL-12p40$^+$ upon LPS stimulation (Fig 3, *A* and *B*). As expected,[9,10] TSLP conditioning significantly inhibited this effect. Conditioning with peanut or HDM also limited DC IL-12p40 production to the same extent as TSLP (Fig 3, *A-C*). These effects were not due to allergen-elicited TSLP production from DCs[37] because culture supernatants did not have detectable levels of TSLP (data not shown). These data indicate that promotion of T_H2 responses by IL-12p40 inhibition can take place after DC contact with either TSLP or the allergens HDM or peanut.

We next investigated whether common allergens could also functionally mimic TSLP induction of OX40L on DCs *in vitro*. We found that vesicular stomatitis virus (VSV) infection of DCs strongly induced OX40L; thus we used this as a positive control. Compared with unstimulated DCs, incubation with HDM or peanut allergens significantly increased the proportion of CD11c$^+$OX40L$^+$ cells and the median fluorescence intensity of OX40L in a manner comparable with TSLP (Fig 3, *D-F*). Again, levels of TSLP upon HDM or peanut stimulation were undetectable (data not shown), implying that OX40L induction on DCs was not due to autocrine TSLP signaling. These data indicate that the common allergens HDM and peanut functionally mimic TSLP action on DCs, therefore rendering it redundant during DC conditioning for T_H2 induction.

OX40L plays an important role in allergic responses to HDM and peanut

We next asked whether OX40L might be important for T_H2 induction to HDM or peanut *in vivo*. First, we evaluated OX40L protein expression after 3 days of allergen exposure. Lung OX40L expression was increased upon HDM exposure, with induction on CD11c$^+$CD11b^{hi}MHCII^{hi} myeloid DCs (Fig 4, *A-D*). Peanut plus CT administration induced OX40L in the duodenum (Fig 4, *E*). Accordingly, the proportion of mesenteric lymph node (LN) CD11c$^+$CD11b$^+$MHCII$^+$ DCs expressing OX40L and their median fluorescence intensities were also significantly increased (Fig 4, *F-H*). The frequency of myeloid DCs in the lung and mesenteric LNs after acute allergen exposure increased 2- to 5-fold (see Fig E4 in this article's Online Repository

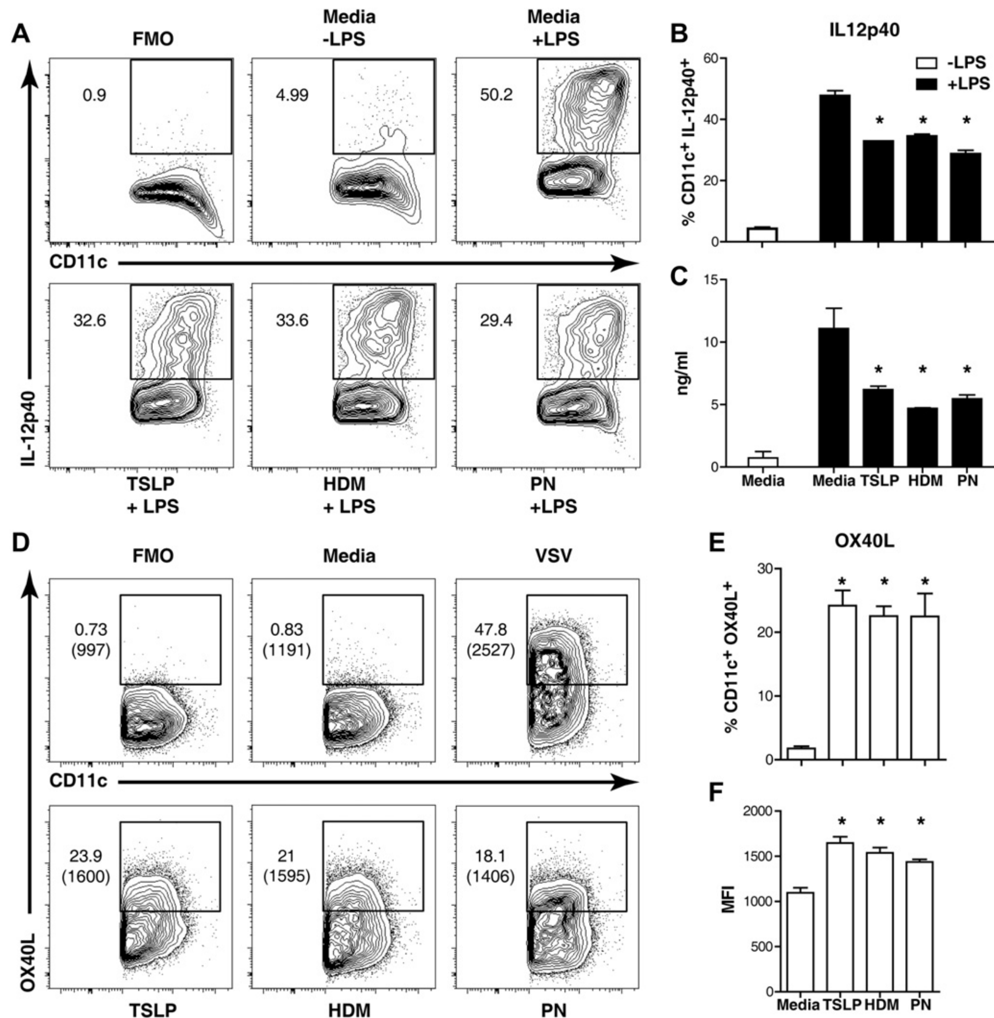

FIG 3. HDM and peanut *(PN)* mimic TSLP in downregulating IL-12p40 and upregulating OX40L. **A-C**, BMDCs were conditioned with media, TSLP, HDM, or peanut and then stimulated with media or LPS. Representative plots (Fig 3, *A*) and quantification (Fig 3, *B*) of $CD11c^+IL\text{-}12p40^+$ cells. Fig 3, *C*, IL-12p40 in culture supernatants. **D-F**, BMDCs were stimulated with media, VSV positive control, TSLP, HDM, or peanut. Representative plots (median fluorescence intensity in brackets; Fig 3, *D*), quantification of $CD11c^+OX40L^+$ cells (Fig 3, *E*), and OX40L median fluorescence intensity (Fig 3, *F*). Shown are 2 to 3 experiments performed in triplicate. **P* < .05 versus media plus LPS (Fig 3, *B* and *C*) or media only (Fig 3, *E* and *F*). *FMO*, Fluorescence minus one.

at www.jacionline.org). Thus sensitization to HDM or peanut plus CT is associated with OX40L upregulation, especially on DCs.

Mice were treated with blocking anti-OX40L antibody or control immunoglobulin throughout HDM administration to test the importance of OX40L on HDM sensitization *in vivo*. Compared with control immunoglobulin-treated and HDM-exposed mice, serum IgG_1 and IgE levels and B-cell activation were significantly attenuated in mice treated with anti-OX40L antibody (Fig 5, *A-C*). The overall inflammatory response was decreased by about 70%, with eosinophil counts being reduced approximately 90% (Fig 5, *D* and *E*). Lung tissue histology confirmed these findings and, in addition, demonstrated reduced goblet cell responses (Fig 5, *F*). $CD4^+$ T-cell activation and the expression of IL-4 and IL-5, but not IFN-γ or IL-17A, were significantly decreased in the absence of OX40L signaling, suggesting a selective impairment of T_H2 immune responses (Fig 5, *G* and *H*).

Blockade of OX40L during peanut sensitization attenuated peanut-specific IgG_1 and IgE levels (Fig 5, *I* and *J*). Upon peanut challenge, anti-OX40L antibody–treated mice experienced approximately 50% less severe anaphylactic hypothermia and hemoconcentration and had less severe clinical signs compared with control immunoglobulin-treated mice (Fig 5, *K-M*). In addition to impaired B-cell responses, T-cell immunity was also hindered, with less than half as many inflammatory cells and approximately 90% less eosinophils in anti-OX40L antibody–treated mice (Fig 5, *N* and *O*). Production of T_H2, but not T_H1 or T_H17, cytokines was impaired (Fig 5, *P*). Collectively, these results show that HDM and peanut allergen–driven humoral and cellular responses operate in a TSLP-independent but OX40L-dependent manner.

IL-25 and IL-33 induce OX40L on DCs *in vitro*

Because mucosal allergen exposure results in TSLP production, we next asked whether other epithelium-associated cytokines with T_H2 induction capacity analogous to TSLP, namely IL-25 and IL-33, were produced during HDM or peanut sensitization. After 3

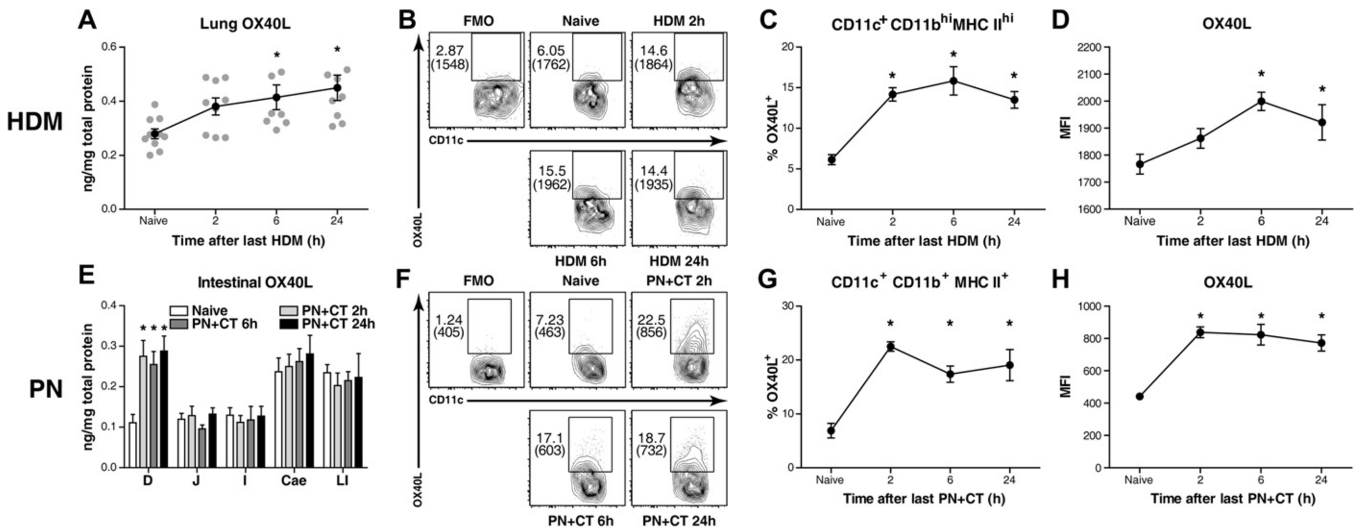

FIG 4. HDM and peanut *(PN)* sensitization induce OX40L on DCs *in vivo*. Mice were exposed to HDM intranasally (**A-D**) or to peanut plus CT intragastrically (**E-H**) for 3 days. Fig 4, *A* and *E*, Kinetics of OX40L induction. Representative plots (median fluorescence intensity in brackets) of OX40L on lung $CD11c^+CD11b^{hi}MHCII^{hi}$ DCs (Fig 4, *B*) or mesenteric LN $CD11c^+CD11b^+MHCII^+$ DCs (Fig 4, *F*). Fig 4, *C*, *D*, *G*, and *H*, Quantification of OX40L induction on DCs. Shown are 2 to 3 experiments with 5 to 6 mice per group per experiment. $^*P < .05$ versus naive. *Cae*, Caecum; *D*, duodenum; *FMO*, fluorescence minus one; *I*, ileum; *J*, jejunum; *LI*, large intestine.

days of HDM exposure, lung IL-25 and IL-33 were induced with slightly different kinetics (Fig 6, *A* and *C*). Similarly, acute peanut plus CT exposure increased duodenal levels of IL-25 and IL-33 (Fig 6, *B* and *D*). These cytokines were not detected in the serum (data not shown), suggesting local rather than systemic influence. They were also not increased in the bronchoalveolar lavage fluid or intestinal washes of allergen-exposed mice (not shown), suggesting that basolateral rather than apical secretion of these cytokines was associated with allergic sensitization.

Because upregulation of IL-25 and IL-33 in allergen-exposed tissues (Fig 6, *A-D*) preceded or was coincident with increased DC OX40L expression (Fig 4), we next evaluated whether IL-25 and IL-33, like TSLP, also induced DC OX40L *in vitro*. DCs have been previously shown to be responsive to IL-33[38] and IL-25.[39,40] Here we show that these cytokines were equally capable of inducing OX40L on DCs *in vitro* (Fig 6, *E* and *F*). TSLP levels were undetectable in all cultures that did not receive TSLP; likewise, IL-25 and IL-33 were undetectable in all cultures that did not receive IL-25 or IL-33 (data not shown). These data suggest that allergen exposure results in the elaboration of multiple epithelium-associated cytokines that converge on DC maturation, including OX40L upregulation. Consequently, we considered whether IL-25, IL-33, or both were important for the induction of allergic disease *in vivo*.

IL-25 and its receptor are dispensable for pulmonary and gastrointestinal allergic sensitization

IL-25 signaling requires the presence of its cytokine-binding receptor subunit, IL-17RB,[14] and exogenous recombinant IL-25 results in severe T_H2 pathology in the lung and gut.[13] Hence to investigate the requirement of IL-25 signaling on the induction of T_H2 responses to HDM and peanut, we attempted to induce allergic asthma or peanut allergy in IL-17RB KO mice.

Compared with saline-exposed mice, HDM-exposed WT or IL-17RB KO mice had similarly increased HDM-specific IgG_1 and total IgE levels and, accordingly, B-cell activation, suggesting equivalent sensitization (Fig 7, *A-C*). Total inflammatory cell numbers, proportions and numbers of eosinophils, and goblet cell responses were comparable between the HDM-exposed WT and IL-17RB KO groups (Fig 7, *D-F*). In regard to T-cell immunity, $CD69^+CD4^+$ T cells and *ex vivo* HDM-specific cytokine production were equally increased to greater than that seen with saline controls in both WT and IL-17RB KO mice (Fig 7, *G* and *H*). Thus T_H2 responses to HDM can fully develop in the absence of IL-25 signaling.

Similar to pulmonary T_H2 responses to HDM, gastrointestinal sensitization of WT and IL-17RB KO mice to peanut resulted in indistinguishably increased peanut-specific IgG_1 and IgE levels above those seen in corresponding naive mice (Fig 7, *I* and *J*). Upon peanut challenge, both groups of peanut-sensitized mice had similar anaphylactic responses (Fig 7, *K-M*). Deficiency of IL-25 signaling also did not impair T-cell priming, as evidenced by peritoneal inflammation and cytokine production (Fig 7, *N-P*).

Treatment of mice with an anti–IL-25 antibody before and throughout sensitization to HDM or peanut resulted in data similar to those generated by using IL-17RB KO mice (see Fig E5 in this article's Online Repository at www.jacionline.org). Therefore although IL-25 is capable of inducing OX40L *in vitro*, these data comprehensively show that IL-25 signaling is not required to generate B- and T-cell allergic immunity to HDM or peanut *in vivo*.

IL-33 signaling mediates allergy to peanut and HDM

Because neither TSLP nor IL-25 played a significant role in initiating HDM or peanut allergic phenotypes, we investigated the relevance of IL-33, which binds the ST2 receptor.[15] Compared with HDM-exposed WT mice, serum HDM-specific IgG_1 levels were significantly decreased and IgE levels were diminished by more than 50% in ST2 KO mice (Fig 8, *A* and *B*). Similarly, the extent of airway and lung inflammation and mucus production

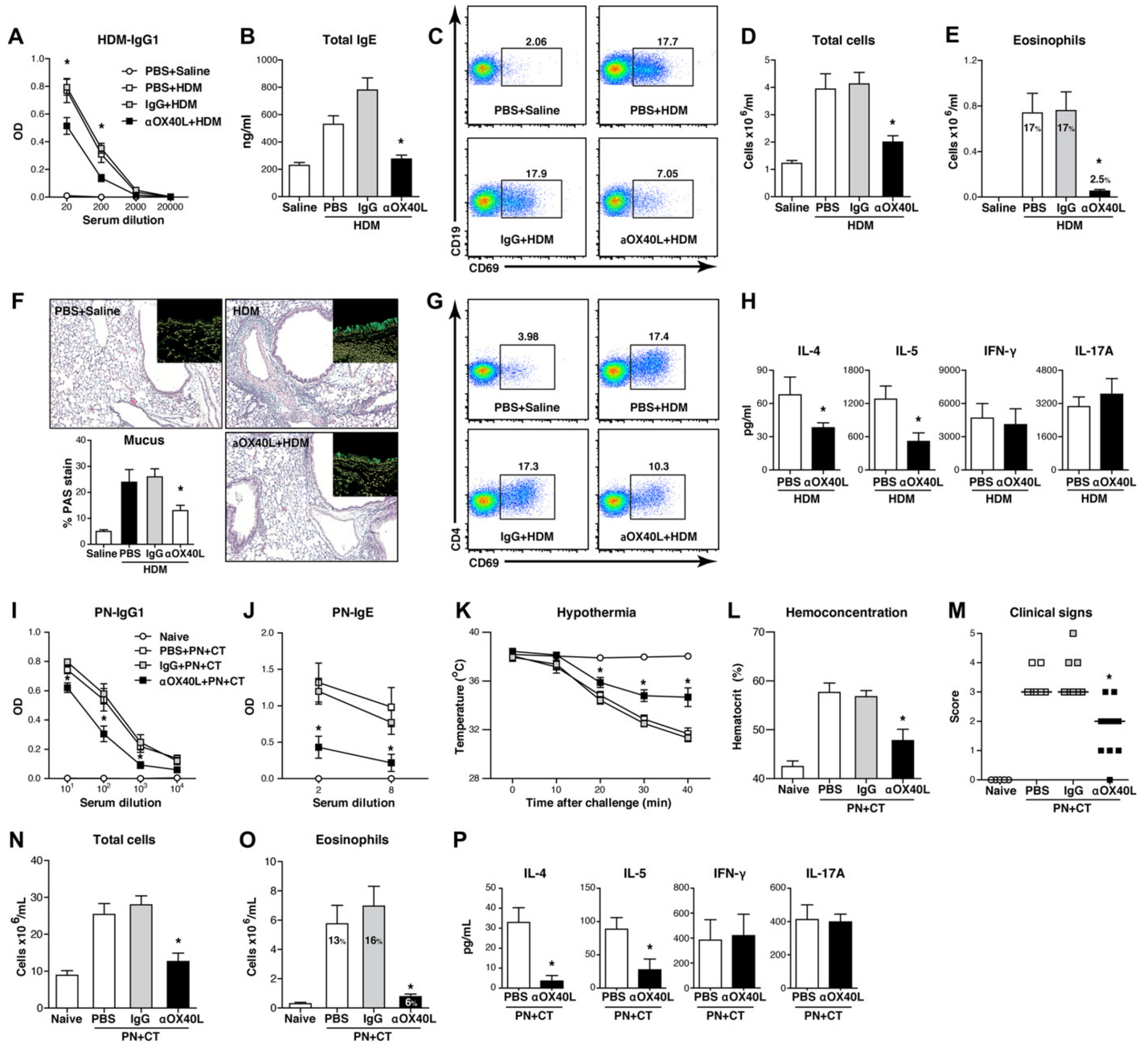

FIG 5. OX40L is required for allergic sensitization to HDM and peanut *(PN)*. **A-H**, Mice received PBS vehicle, IgG, or anti-OX40L before and throughout 10 days of saline or HDM exposure. HDM-specific IgG_1 (Fig 5, *A*) and total IgE (Fig 5, *B*) levels in serum. Fig 5, *C*, Representative CD69 expression by lung $CD45^+CD3^-CD19^+$ B cells. Total cell (Fig 5, *D*) and eosinophil (Fig 5, *E*) counts in bronchoalveolar lavage fluid; mean percentages of eosinophils are shown in *bars*. Fig 5, *F*, Representative lung histology stained with hematoxylin and eosin (×100 magnification) or periodic acid–Schiff *(PAS)* insets (×400 magnification). HDM exemplifies similar sections from PBS plus HDM and IgG plus HDM groups. Periodic acid–Schiff quantification is shown at *bottom left*. Fig 5, *G*, Representative CD69 expression by lung $CD45^+CD3^+CD4^+$ T cells. Fig 5, *H*, Cytokine production by HDM-stimulated splenocytes. **I-P**, Mice received PBS, IgG, or anti-OX40L during peanut sensitization and were challenged 1 week later. Serum peanut-specific IgG_1 (Fig 5, *I*) and IgE (Fig 5, *J*) levels 1 day before challenge. Fig 5, *K-M*, Anaphylactic assessment. Total (Fig 5, *N*) and eosinophil (Fig 5, *O*) cell counts in peritoneal lavage; mean percentages of eosinophils are shown in *bars*. Fig 5, *P*, Cytokine production by peanut-stimulated splenocytes. Shown are 3 to 8 mice per group per experiment. *$P < .05$ versus PBS or IgG mice exposed to HDM or peanut plus CT.

was lower in HDM-exposed ST2 KO mice, with the infiltration of eosinophils decreased by more than 80% (Fig 8, *C-E*). The proportion of activated $CD4^+$ T cells in the ST2 KO mice was dramatically decreased, as was the *ex vivo* production of IL-4, IL-5, IFN-γ, and IL-17A (Fig 8, *F* and *G*), suggesting an overall impairment in adaptive immune priming.

In response to the peanut sensitization regimen, peanut-specific IgG_1 and IgE levels were diminished by more than 80% in ST2

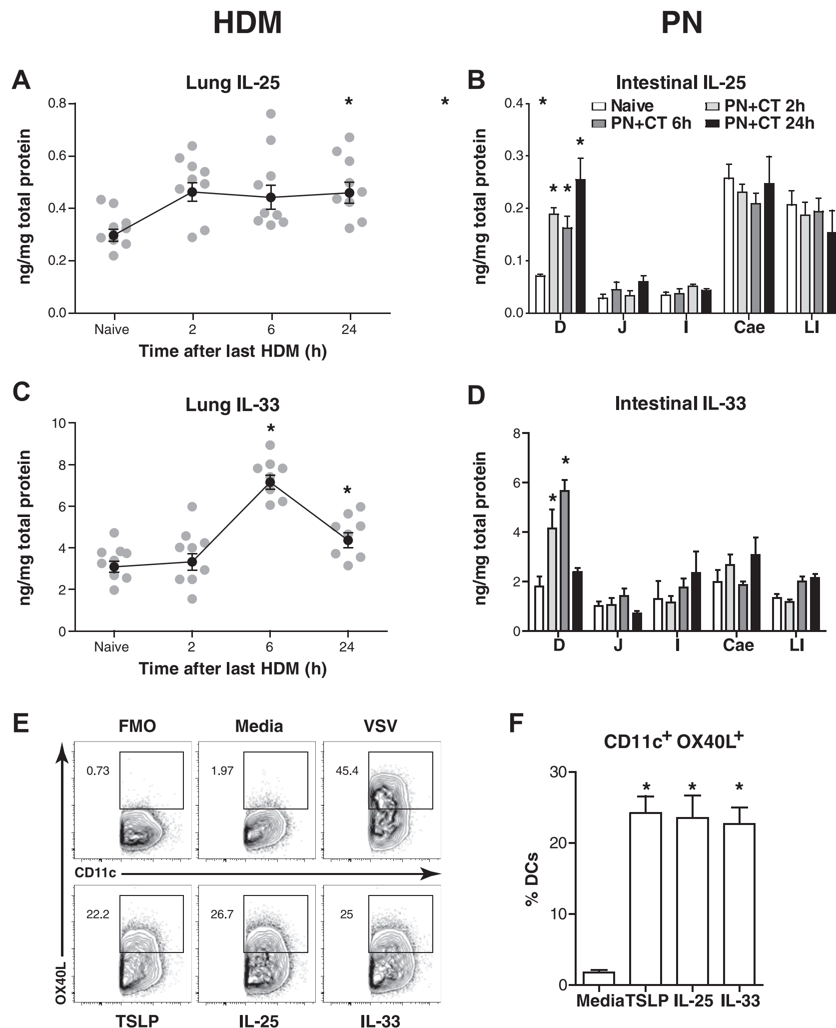

FIG 6. IL-25 and IL-33 induce OX40L on DCs *in vitro*. Kinetics of IL-25 (**A** and **B**) and IL-33 (**C** and **D**) induction after 3 days of HDM or peanut *(PN)* plus CT. Representative plots **(E)** and quantification **(F)** of CD11c$^+$OX40L$^+$ BMDCs stimulated with media, VSV, recombinant TSLP, IL-25, or IL-33. Two experiments were performed with 5 to 6 mice per group per experiment (Fig 6, *A-D*) or replicates of 2 to 4 (Fig 6, *E* and *F*). $*P < .05$ versus naive or media. *Cae*, Caecum; *D*, duodenum; *FMO*, fluorescence minus one; *I*, ileum; *J*, jejunum; *LI*, large intestine.

KO mice compared with those seen in WT control animals (Fig 8, *H* and *I*). This translated into protection from anaphylactic shock on peanut challenge because these mice did not exhibit hypothermia and had 50% less hemoconcentration and minor clinical signs of anaphylaxis compared with those seen in sensitized and challenged WT mice (Fig 8, *J-L*). Eosinophilic inflammation in peanut plus CT–exposed ST2 KO mice was markedly reduced by about 95%, suggesting severely inhibited T_H2 priming (Fig 8, *M* and *N*). In agreement, T_H2 cytokine production *ex vivo* was also significantly decreased in ST2 KO mice (Fig 8, *O*).

Although IL-33 has been reported to play a role during the effector phase of passive anaphylaxis to 2,4-dinitrophenol,[41] we did not find IL-33 to drive the anaphylactic event in our model of active anaphylaxis to peanut. ST2-Fc treatment only during the sensitization phase ameliorated anaphylaxis because of reduced peanut-specific IgE production (see Fig E6, *A-D*, in this article's Online Repository at www.jacionline.org); treatment before induction of anaphylaxis[41] had no effect on hypothermia or hemoconcetration (see Fig E6, *E-H*). These data point toward a central role for IL-33 during sensitization, rather than the effector phase of peanut allergy. Collectively, these data show that unlike TSLP and IL-25, IL-33 plays a nonredundant role in the initiation of respiratory (HDM) and food (peanut) allergy.

Allergen-elicited IL-33 potently induces DC OX40L and ILCs *in vivo*

We observed that TSLP, IL-25, and IL-33 could induce OX40L *in vitro*, yet only IL-33 was required for the generation of T_H2 immunity to HDM or peanut *in vivo*. To better understand how DCs responded to these cytokines *in vivo*, mice received 0.5 μg of recombinant TSLP, IL-25, or IL-33 intranasally for 3 days, and 24 hours later, tissues were analyzed.[12,14,42] In the lung the levels of total and DC OX40L were increased in response to IL-33 but not TSLP or IL-25 (Fig 9, *A* and *B*), suggesting that OX40L induction *in vivo* is exquisitely sensitive to IL-33.

Correlating with OX40L induction, we also observed the expansion of a lineage-negative population (CD45$^+$Lin$^-$;

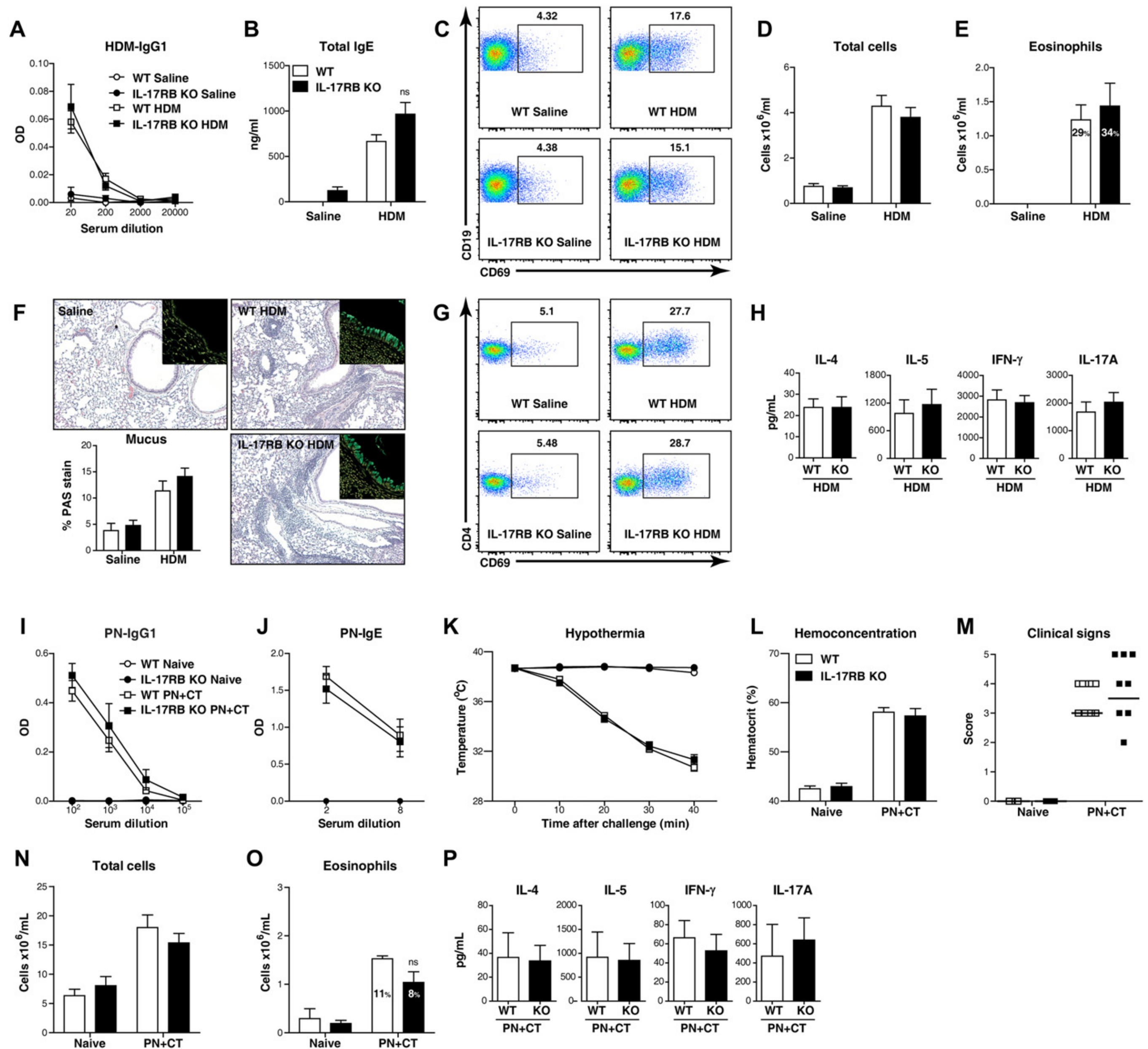

FIG 7. IL-17RB is dispensable for allergic sensitization to HDM and peanut *(PN)*. **A-H**, WT and IL-17RB KO mice were exposed to 10 days of HDM. Serum HDM-specific IgG_1 (Fig 7, *A*) and total IgE (Fig 7, *B*) levels. Fig 7, *C*, Representative lung $CD45^+CD3^-CD19^+$ B-cell expression of CD69. Total (Fig 7, *D*) and eosinophil (Fig 7, E) counts in bronchoalveolar lavage fluid; mean percentages are shown in *bars*. Fig 7, *F*, Representative lung histology stained with hematoxylin and eosin (×100 magnification) or periodic acid–Schiff (PAS; ×400 magnification) insets. Saline exemplifies similar sections from the WT saline and IL-17RB KO saline groups. Periodic acid–Schiff analysis is shown at *bottom left*. Fig 7, *G*, Expression of CD69 by lung $CD45^+CD3^+CD4^+$ T cells. Fig 7, *H*, Cytokine production by HDM-stimulated splenocytes. **I-P**, WT or IL-17RB KO mice were sensitized to peanut and challenged intraperitoneally 1 week later. Serum peanut-specific IgG_1 (Fig 7, *I*) and IgE (Fig 7, *J*) levels 1 day before challenge. Fig 7, *K-M*, Anaphylactic assessment. Total (Fig 7, *N*) and eosinophil (Fig 7, *O*) cell counts in peritoneal lavage fluid; mean percentages are shown in *bars*. Fig 7, *P*, Cytokine production by peanut-stimulated splenocytes. Shown are 2 experiments with 2 to 8 mice per group per experiment.

$CD3^-CD19/B220^-CD11c^-CD11b^-$) expressing c-kit, Sca-1, and Thy1.2 (CD90.2) after administration of IL-33 but not TSLP or IL-25 (Fig 9, *C* and *D*). Expression of ST2 and inducible costimulator was present on 30% to 50% of these cells (data not shown). Recent reports describe these novel cells as ILCs, which can respond to IL-25, IL-33, or both and expand on airway viral or gastrointestinal helminth infection.[17] Increased OX40L expression and expansion of ILCs was observed not only in the lung but also in the thoracic LNs, small intestine, and mesenteric LNs upon intraperitoneal IL-33 (data not shown). These data suggest that T_H2 induction to HDM or peanut relies on the ability of IL-33 to upregulate OX40L and expand ILCs. Accordingly, the

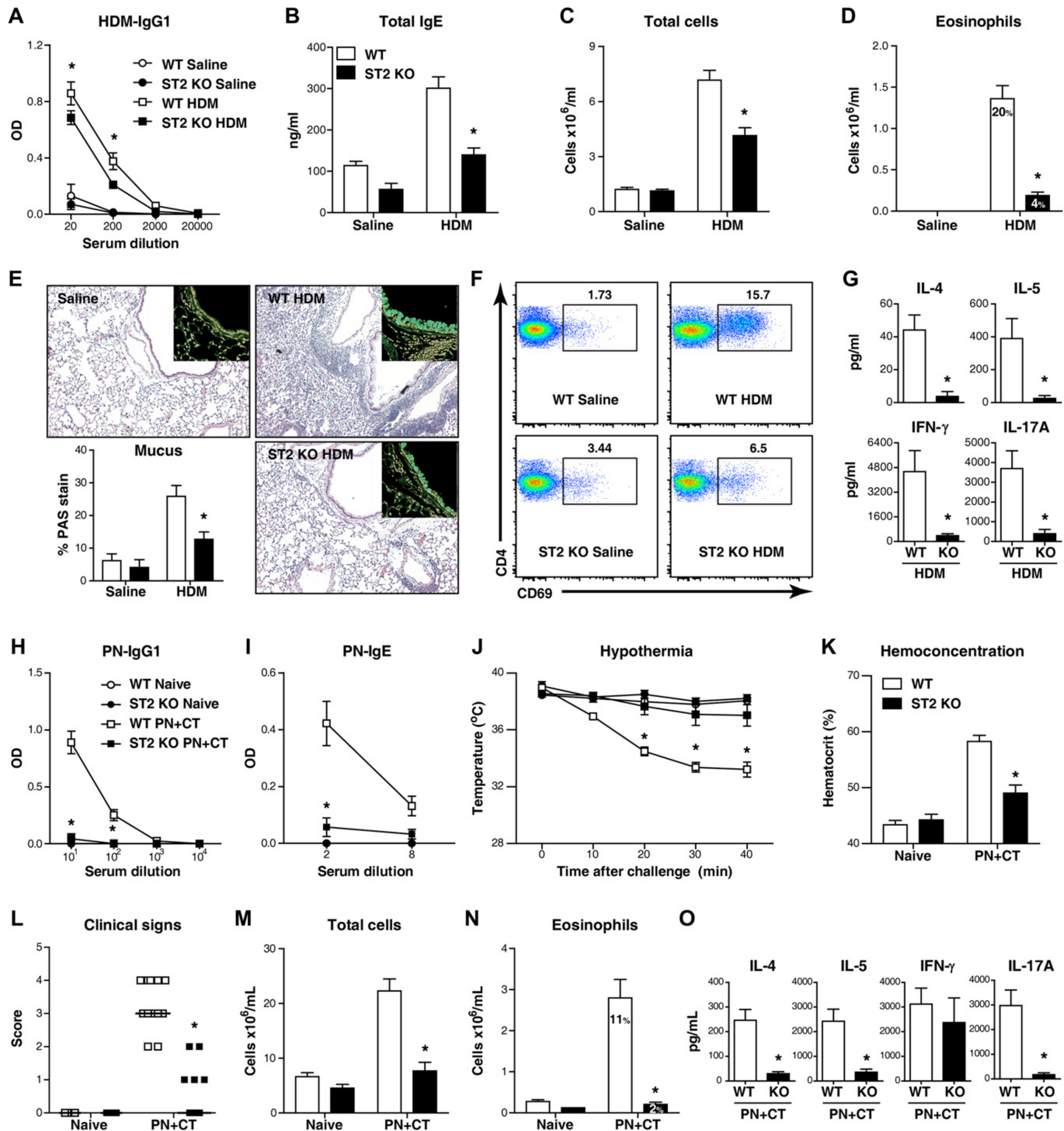

FIG 8. The IL-33 receptor ST2 is required for allergic sensitization to HDM and peanut *(PN)*. **A-G**, WT and ST2 KO mice were exposed to 10 days of HDM. Serum HDM-specific IgG_1 (Fig 8, *A*) and total IgE (Fig 8, *B*) levels. Total (Fig 8, *C*) and eosinophil (Fig 8, *D*) counts in bronchoalveolar lavage fluid; mean percentages are shown in *bars*. Fig 8, *E*, Representative lung histology stained with hematoxylin and eosin (×10 magnification) or periodic acid–Schiff (*PAS*; ×40 magnification) insets. Saline exemplifies similar sections from the WT saline and ST2 KO saline groups. Periodic acid–Schiff analysis is shown at *bottom left*. Fig 8, *F*, Expression of CD69 by lung $CD45^+CD3^+CD4^+$ T cells. Fig 8, *G*, Cytokine production from HDM-stimulated splenocytes. **H-O**, WT or ST2 KO mice were sensitized to peanut and then challenged intraperitoneally 1 week later. Serum peanut-specific IgG_1 (Fig 8, *H*) and IgE (Fig 8, *I*) levels 1 day before challenge. Fig 8, *J-L*, Anaphylactic assessment. Total (Fig 8, *M*) and eosinophil (Fig 8, *N*) cell counts in peritoneal lavage fluid; mean percentages are shown in *bars*. Fig 9, *O*, Cytokine production by peanut-stimulated splenocytes. Shown are 2 experiments with 2 to 8 mice per group per experiment. $^*P < .05$ versus WT mice exposed to HDM or peanut plus CT.

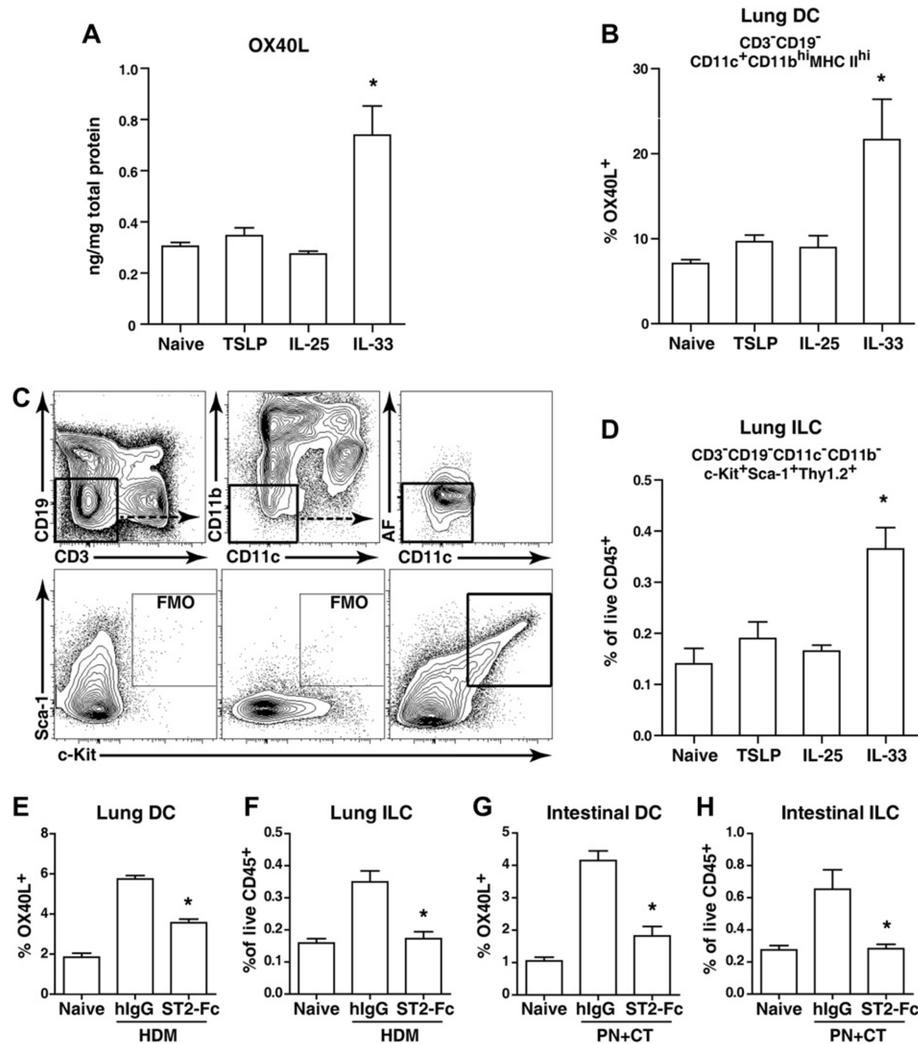

FIG 9. HDM and peanut *(PN)* induce DC OX40L and ILCs *in vivo* through IL-33. Recombinant TSLP, IL-25, or IL-33 (**A-D**) or HDM or peanut plus CT (**E-H**) was administered intranasally or intragastrically for 3 days. Fig 9, *A*, OX40L normalized to total protein in lung homogenates. Fig 9, *B*, Quantification of DC OX40L induction. Fig 9, *C*, Gating strategy for ILCs. Fig 9, *D*, Quantification of ILC expansion. Fig 9, *E-H*, Effect of ST2-Fc or IgG on DC OX40L and ILC induction. Shown are 2 to 3 experiments with 2 to 5 mice per group per experiment. **P* < .05 versus naive, TSLP, or IL-25 groups (Fig 9, *A-D*) or allergen-exposed immunoglobulin-treated mice (Fig 9, *E-H*). *AF*, Autofluorescence; *FMO*, fluorescence minus one.

proportion of lung and small intestinal $OX40L^+$ DCs and ILCs was abrogated in allergen-exposed mice treated with ST2-Fc compared with those treated with control immunoglobulin (Fig 9, *E-H*). IL-33, more so than TSLP or IL-25, is therefore central to HDM- or peanut-mediated upregulation of OX40L and expansion of ILCs during allergic sensitization.

DISCUSSION

It has been increasingly recognized that T_H2 immunity results from adaptive immune responses that are shaped by initial innate signals. Among these, epithelium-associated cytokines, such as TSLP, IL-25, and IL-33, have been the subject of intense investigation. Here we investigated the initiation of allergic asthma and food allergy using models involving 2 clinically relevant allergens and different mucosal sites and sensitization strategies. We show that the development of HDM and peanut allergy, both of which evolve through canonical T_H2 pathways (see Fig E3),[24] is TSLP and IL-25 independent and, conversely, requires IL-33 for OX40L induction, ILC expansion, and, ultimately, disease manifestation. We used established KO mice, with their proper strain-matched control animals, as well as neutralizing antibody strategies to validate our conclusions.

Studies in models of allergic inflammation[26,27] have led to the proposition that TSLP is a master regulator of allergic responses.[22,35] Notably, sensitization in these studies was achieved through exposure of a nonmucosal site (eg, peritoneal cavity) to the model antigen OVA adsorbed to aluminum-based adjuvants. At variance, our data in the HDM model demonstrate that the extent of allergic sensitization, airway eosinophilic inflammation, and lung pathology was comparable in the presence or absence of TSLP signaling. Furthermore, peanut sensitization, systemic anaphylactic responses, and eosinophilic inflammation were intact in the absence of TSLP, its receptor or basophils. Similarly, anaphylaxis was not prevented when using OVA instead of peanut, which is in accordance with a recent report showing intact

OVA plus CT–induced anaphylaxis in TSLPR-deficient mice.[43] Thus the requirement for TSLP in generating T_H2 immunity to allergens is not as pervasive as originally proposed. To our knowledge, TSLP-independent T_H2 responses have only been reported in helminth infection systems.[8,44]

The ability of TSLP to induce T_H2 responses has been mainly attributed to inhibition of IL-12 production and upregulation of OX40L on DCs.[8,10,11] Here we show that HDM and peanut inhibited DC IL-12 production similarly to TSLP, thus making it redundant. This concept is in agreement with data suggesting that TSLP-independent antihelminth responses are due to helminth excretory-secretory products that supplant TSLP inhibition of DC IL-12.[8] Furthermore, HDM and peanut also mimicked TSLP induction of OX40L on DCs. Thus our data identify common allergens (HDM and peanut) that mimic TSLP actions on DCs, resulting in the induction of T_H2 responses independently of TSLP.

In OVA peptide–pulsed DC/T-cell cocultures, Blázquez and Berin[45] suggested a critical role for OX40L in T_H2 cytokine production. Here we found that allergen exposure induced OX40L on DCs *in vitro*, as well as *in vivo*, in the lung and duodenum. Neutralization of OX40L significantly impaired HDM or peanut sensitization, with effects similar to those reported recently in HDM-exposed OX40 KO mice.[46] Thus our work shows that these models of HDM and peanut allergy originate from a TSLP-independent but OX40L-dependent pathway.

We (Fig 6) and others[25,47] have shown increased levels of IL-25 and IL-33 in the lung after HDM exposure. Here we also report that these cytokines are produced in the duodenum after peanut plus CT exposure. TSLP and IL-33 have been shown to induce OX40L on DCs *in vitro*.[11,48] We confirm these data and further demonstrate that IL-25 can upregulate OX40L to a similar extent as TSLP and IL-33. Consequently, these data indicate that multiple epithelium-associated cytokines are elaborated upon allergen exposure to the respiratory and gastrointestinal mucosae, all converging on, at least, OX40L upregulation on DCs for T_H2 induction.

Although exogenous IL-25 is capable of inducing T_H2 responses,[13] we found no significant effect on immunoglobulin, cytokine, and inflammatory parameters when we blocked IL-25 during HDM or peanut sensitization or examined IL-17RB KO mice. These findings are at variance with previous reports using experimental models of intraperitoneal OVA plus alum–induced asthma in which blockade of IL-25 significantly reduced T_H2 cytokine production and eosinophilic inflammation.[49] IL-25 has also been implicated in promoting T_H2 immunity in host defense against some helminths that infect the gut.[50,51] Thus although IL-25 is increasingly considered key in the regulation of T_H2 immunity,[52] our data clearly indicate that IL-25 is not necessary for T_H2 priming and subsequent effector responses to allergens, such as HDM and peanut.

With respect to IL-33, Coyle et al[53] initially showed a critical role for ST2, which was later identified as the IL-33 receptor, in both T_H2 cellular and humoral responses in an OVA model.[53] However, subsequent studies yielded variable conclusions on the importance of IL-33 for T_H2 responses.[54,55] Our data demonstrate that IL-33 signaling is required for T_H2 priming to both HDM and peanut. These effects translated into significantly less lung pathology and anaphylaxis and were associated with a superior ability of IL-33, over TSLP or IL-25, to increase OX40L levels, particularly on DCs, *in vivo*. In addition, IL-33 markedly expanded a population of ILCs, which have recently been identified and shown to be capable of producing T_H2 cytokines.[17] Although ILCs were reported to be expanded during secondary responses to OVA,[56] we report, for the first time, that allergic sensitization to HDM and peanut are associated with ILC expansion and that this is IL-33 dependent.

TSLP, IL-25, and IL-33 are typically recognized as epithelium-derived cytokines, but other cell types are able to produce these cytokines, particularly in chronic inflammatory states. The specific cellular sources, targets, and functional effects of these cytokines on chronic allergic disease severity, progression, and/or exacerbation are only beginning to be investigated. For example, IL-25 has recently been shown to be associated with collagen deposition, lung dysfunction, or both in atopic patients and mice,[57,58] and Oboki et al[42] have generated IL-33 KO mice and suggested IL-33 to be important in the maintenance of airway inflammation in a model of chronic HDM exposure.

Our data, along with those of previous reports using other experimental systems, reveal that TSLP, IL-25, and IL-33 are differentially required to generate T_H2 responses. For example, antihelminth host defense to *Trichuris muris* is dependent on TSLP, IL-25, and IL-33, whereas *Nippostrongylus brasiliensis* requires IL-25 but not TSLP or IL-33.[8,10,50,54,59] In the case of allergic responses, OVA plus alum–driven T_H2 initiation is dependent on all of these cytokines (Fig E1, *G-K*),[26,27,49,53] whereas HDM- and peanut plus CT–induced responses rely on IL-33 but not TSLP or IL-25. Clearly, diverse antigen-host interactions are able to launch distinct molecular programs highlighting a diversity of pathways leading to T_H2 immunity. This might provide a mechanistic basis for the growing clinical recognition that allergic diseases are heterogeneous. Whether these clinicopathologic variants/endotypes are a result of different subtypes of T_H2 cells (eg, TSLP driven, IL-25 driven, and/or IL-33 driven), each with their own molecular signature and effector armamentarium and, ultimately, distinct therapeutic susceptibilities, remains to be explored.

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Key messages

- **TSLP and IL-25 are redundant but IL-33 is central in the initiation of T_H2 responses to the common allergens HDM and peanut.**
- **IL-33–mediated allergic sensitization to these airway and food allergens requires DC OX40L and is associated with expansion of ILCs.**

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METHODS

Sensitization and treatment protocols

HDM administration. Twenty-five micrograms of HDM extract (Greer Laboratories, Lenoir, NC) in 10 μL of saline was administered intranasally to isoflurane-anesthetized mice for 3 or 10 consecutive days.[E1,E2] Mice were killed 24 hours after the last exposure, unless otherwise specified.

Peanut plus CT administration. One milligram of peanut (Kraft, Northfield, Ill) with 10 μg of CT (List Biological Laboratories, Campbell, Calif) in PBS was gavaged intragastrically by using 12-gauge, 2.5-inch intragastric feeding needles (DELVO SA, Biel/Bienne, Switzerland) daily for 3 consecutive days or weekly for 4 weeks.[E3-E5] In some experiments 1 mg of OVA was gavaged instead of peanut, or the route of administration was intraperitoneal instead of intragastric.

OVA or peanut and alum administration. Fifty micrograms of OVA (grade V; Sigma, St Louis, Mo) emulsified in 1.3 mg of aluminum hydroxide (Sigma) was administered intraperitoneally on days 1 and 14.[E6] Anesthetized mice were challenged intranasally with 150 μg of OVA in 40 μL of PBS for 4 days, starting from day 21. Mice were analyzed 24 hours after the last challenge. In some experiments peanut was used instead of OVA, and mice were challenged intraperitoneally with 500 μg of peanut instead of intranasally to induce anaphylaxis and inflammation.

Antibody administration. The following antibodies and corresponding controls (rat, rabbit, or human IgG; Sigma) in PBS were administered intraperitoneally either 24 hours before the first HDM exposure and every 4 to 5 days of the HDM protocol or 24 hours before each peanut sensitization: 1 mg of anti-TSLP or anti–IL-25 (Amgen, with neutralizing activity[E7,E8]), 250 μg of ST2-Fc (R&D Systems, Minneapolis, Minn), 200 or 400 μg of anti-OX40L (R&D Systems), 100 μL anti-asialo GM1 (Wako, Richmond, Va), or 100 μg of intravenous Ba103 (Hycult Biotech, Uden The Netherlands). In some experiments 25 μg of ST2-Fc was administered intranasally.

Cytokine administration. Recombinant mouse TSLP, IL-25, or IL-33 (0.5 μg, R&D systems or eBioscience [San Diego, Calif]) was administered intranasally or intraperitoneally daily for 3 days, and tissues were analyzed 24 hours later.

Induction and grading of peanut-induced anaphylaxis

One to 2 weeks after the last sensitization, 500 μg of peanut (Greer Laboratories) or OVA in PBS was injected intraperitoneally. Mice were monitored for 40 minutes after challenge for changes in rectal (core) body temperature (VWR International, West Chester, Pa). Forty minutes after challenge, heparin-anticoagulated blood was centrifuged at 6000 to 6200 rpm for 1 minute to measure hematocrit. Clinical signs were evaluated as follows: 0, no clinical signs; 1, pruritis (repetitive hind leg ear canal scratching); 2, periorbital/periauricular edema, piloerection; 3, lethargy/decreased activity, lying prone; 4, no response to whisker provocation; and 5, end point (seizure or death).

Collection of specimens

Peripheral blood was collected, and serum was stored at −20°C. Lungs underwent lavage with PBS containing complete protease inhibitor (Roche, Mannheim, Germany). Total cell counts were determined by using a hemocytometer. Cytospin preparations were stained with the Protocol Hema 3 stain set (Fisher Scientific, Hampton, NH), and differential cell counts were determined by using standard hemocytologic criteria. In some cases the left lung was slowly inflated and fixed with 10% formalin for histology.

Seventy-two hours after anaphylaxis, peritoneal lavage was performed with 10 mmol/L ice-cold EDTA in PBS.

Tissue homogenization

Samples were collected in 500 μL of PBS containing complete protease inhibitor (Roche), rotor-stator homogenized (Polytron; Kinematica, Lucerne, Switzerland), incubated with 1% Triton-X100 for 40 minutes at 4°C, and spun, and supernatants were spun again (12,000 rpm for 10 minutes at 4°C). Supernatants were collected, and protein content was quantified by using the Bradford assay (Bio-Rad Laboratories, Hercules, Calif) and stored at −80°C.

Cytokine and immunoglobulin analysis

Cytokines were measured by using ELISA (kits from eBioscience and R&D Systems) or Luminex assay (multiplex kits from Millipore, Temecula, Calif). Total or antigen-specific immunoglobulin levels were measured by using sandwich ELISA.[E1,E4,E5,E9] Antigen-specific immunoglobulin ELISAs are depicted as OD – background, where background = 2*(SD + Average of at least 20 blank wells).

BMDC generation and stimulation

GM-CSF–derived BMDCs were generated[E10] and then incubated with media, HDM, crude peanut extract, 100 ng/mL recombinant mouse (rm) TSLP (R&D Systems), rmIL-25 (R&D Systems), and rmIL-33 (R&D Systems) for 16 to 24 hours and analyzed directly or then stimulated with 10 ng/mL *Escherichia coli* 0111:B4 LPS (cell culture tested; Sigma). BMDC viability was greater than 80%. For intracellular cytokine staining, BMDCs were stimulated with or without LPS for 12 hours and brefeldin A (eBioscience) for the last 6 hours. Some BMDCs were infected with 25 multiplicity of infection VSV-ΔM51, which transduces DCs without significant progeny virus production or effect on viability.[E11]

Cell isolation and culture

Lungs. Lungs were chopped into small pieces and then digested with Collagenase type I (Gibco, Invitrogen, Carlsbad, Calif) in HBSS for 1 hour at 37°C before being pressed through 40-μm cell strainers, and RBCs were lysed with ACK buffer. Cells were washed and filtered again before analysis.

Intestines. After flushing intestinal contents with cold PBS, the intestines were opened longitudinally, fat and mucus were removed manually, and the intestines were cut into approximately 1-cm pieces before epithelium and mucus were removed by means of repeated 15-minute 37°C incubations in PBS with 10 mmol/L EDTA, 10 mmol/L HEPES, 1 mmol/L dithiothreitol, and vortexing. Epithelium-free lamina propria was then incubated with 0.25 Wünsch units/mL Liberase (Roche) for 60 minutes with 20 U/mL DNAse I (Roche), pressed through a 40-μm strainer, and washed before analysis.

Spleens. Spleens were pressed through 40-μm strainers and incubated with ACK before being resuspended in complete RPMI (RPMI supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol [Gibco, Invitrogen]). Viable cells (8×10^5) were cultured in triplicate in medium alone or with 15.625 μg/mL HDM or 250 μg/mL crude peanut extract in flat-bottom, 96-well plates (BD, San Jose, Calif). After 5 days, triplicates were pooled, and cell-free supernatants were stored at −20°C.

LNs. LNs were triturated between frosted slides in HBSS, washed, and filtered (40 μm).

Flow cytometry

To minimize nonspecific and Fc receptor–mediated binding, cells were first incubated with anti-CD16/CD32, (2.4G2; eBioscience) in 0.5% to 1% BSA and 10 mmol/L EDTA-supplemented PBS for at least 15 minutes at 4°C. Antibodies were from BD Biosciences, eBioscience, BioLegend (San Diego, Calif), or MD Biosciences (Saint Paul, Minn) and were titrated before use. ST2–fluorescein isothiocyanate (FITC), CD11c–Alexa Fluor (AF) 488, peridinin-chlorophyll-protein complex (PerCP)–Cy5.5, phycoerythrin (PE)–Cy7, or allophycocyanin (APC); OX40L-PE or biotin (eBioscience and Cedarlane [Burlington, NC]); IL-12p40–PE or APC; CD3–PE-Cy5, PerCP-Cy5.5, PE–eFluor 710, PE-Cy7, AF700, or eFluor 450 or V500; CD19–PerCP-Cy5.5, PE-Cy7, or APC-Cy7; c-Kit–PE-Cy5, PE-Cy7, or APC; MHC II (I^A/I^E)–AF700, eFluor 450, or eFluor 650; Sca-1–AF700; CD86-FITC, AF700, or biotin; CD45–APC-Cy7 or eFluor 605; Thy1.2–APC-eFluor 780; CD8–eFluor 450 or PerCP; CD11b–PE-Cy7, AF700, or Brilliant Violet 421; B220–APC-eFluor 780 or V500; CD4-APC, PerCP, eFluor 650, or V500; CD127–PE-Cy7; NK1.1-APC or PE-Cy7; CD49b (DX5)–PE, PE-Cy7 or biotin; CD69-PE or PE-Cy7; IgE-FITC or PE; or inducible

costimulator–FITC were used. Propidium iodide (eBioscience) and forward scatter/side scatter were used to exclude dead cells and doublets. BD Cytofix/Cytoperm was used for intracellular staining. BD CompBeads were used for compensation, except for V500-conjugated antibodies, where cells were stained instead. Data were acquired on a BD LSR II and analyzed with FlowJo software (Tree Star, Ashland, Ore), including software compensation. Fluorescence minus one controls were used for gating.

Histology and morphometric analysis

Lung tissue was embedded in paraffin and cut at a thickness of 4 μm. Sections were stained with hematoxylin and eosin or periodic acid–Schiff. Images of the main airways were analyzed by using a custom system (Northern Eclipse software; Empix Imaging, Mississauga, Ontario, Canada) that calculates the percentage of tissue area that was positively stained within a 30-μm-wide band from the basement membrane extending into the airway lumen.[E9]

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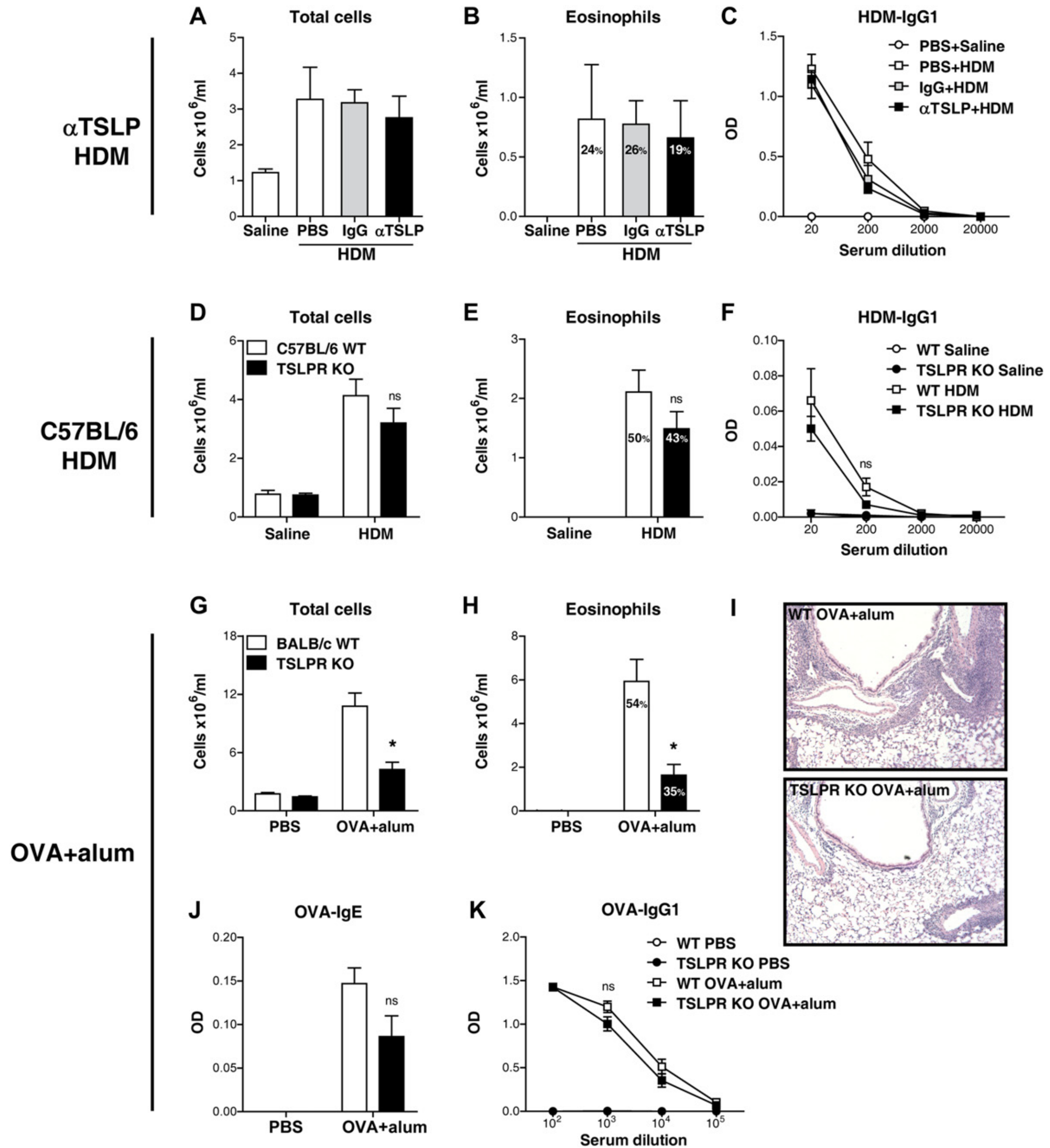

FIG E1. The TSLP-TSLPR axis is differentially required in OVA- and HDM-induced immune-inflammatory responses. **A-C**, BALB/c mice received PBS vehicle, rat IgG, or anti-TSLP blocking antibody 1 day before and throughout the HDM exposure protocol. **D-F**, C57BL/6 WT and TSLPR KO mice were exposed daily to HDM intranasally for 10 days. **G-K**, As previously reported,[E6] BALB/c WT and TSLPR KO mice were sensitized with OVA plus alum intraperitoneally on days 1 and 14 and then challenged with OVA intranasally for 4 days starting on day 21. Total cells (Fig E1, *A*, *D*, and *G*) and eosinophil counts (Fig E1, *B*, *E*, and *H*) in bronchoalveolar lavage fluid. Serum HDM-specific IgG_1 (Fig E1, *C* and *F*) and OVA-specific IgE (Fig E1, *J*) or IgG_1 (Fig E1, *K*) levels. Fig E1, *I*, Representative lung sections stained with hematoxylin and eosin to assess inflammation. One experiment with 2 to 7 mice per group (Fig E1, *A-C*), 2 experiments with 3 to 7 mice per group per experiment (Fig E1, *D-F*), or 2 experiments with 4 to 5 mice per group per experiment (Fig E1, *G-K*). $*P < .05$ versus PBS plus HDM and IgG plus HDM (Fig E1, *A-C*), WT HDM (Fig E1, *D-F*), or WT OVA plus alum (Fig E1, *G-K*). *PN*, Peanut.

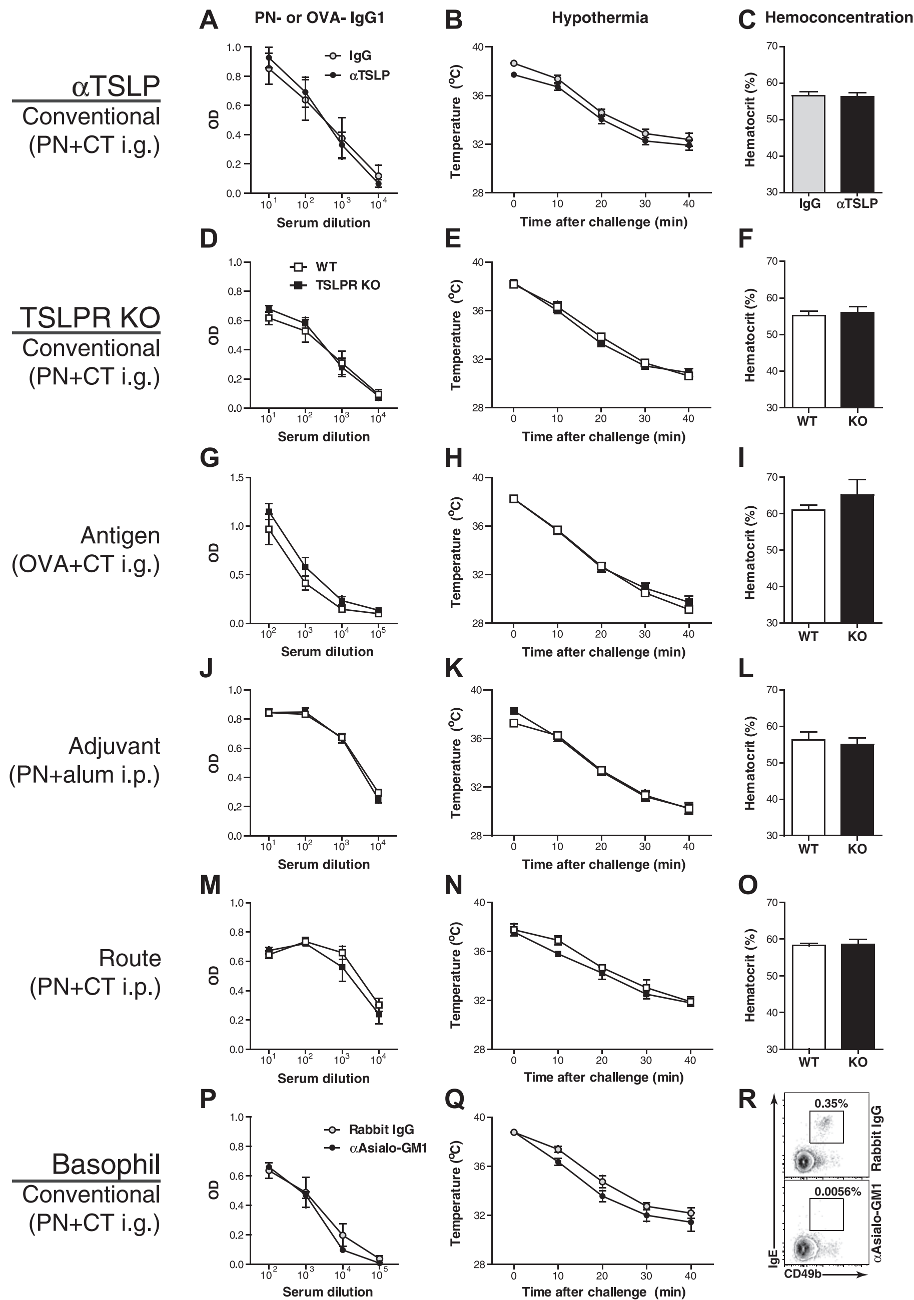

FIG E2. TSLPR independence does not alter with antigen, sensitizing adjuvant, or route of sensitization, and basophils are not required for peanut *(PN)* allergy. WT or TSLPR KO mice were sensitized with peanut plus CT intragastrically (*i.g.*; **A-F** and **P-R**), OVA plus CT intragastrically **(G-I)**, or peanut plus CT intraperitoneally (*i.p.*; **M-O**) and then challenged. Fig E2, *A-C*, Mice received anti-TSLP antibody or rat IgG 1 day before each gavage. **J-L**, Mice were sensitized with peanut plus alum intraperitoneally. Fig E2, *P-R*, Anti-asialo GM1 or rabbit IgG was injected 1 day before each gavage. Fig E2, *A*, *D*, *G*, *J*, *M*, *P*, Serum peanut- or OVA-specific IgG_1 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E2, *B*, *E*, *H*, *K*, *N*, and *Q*) and hematocrit levels (Fig E2, *C*, *F*, *I*, *L*, and *O*). Fig E2, *R*, Proportion of $CD3^{-}CD19^{-}NK1.1^{-}$ c-$Kit^{-}CD49b^{+}IgE^{+}$ basophils in spleens. Shown are 2 experiments with 3 to 8 mice per group per experiment.

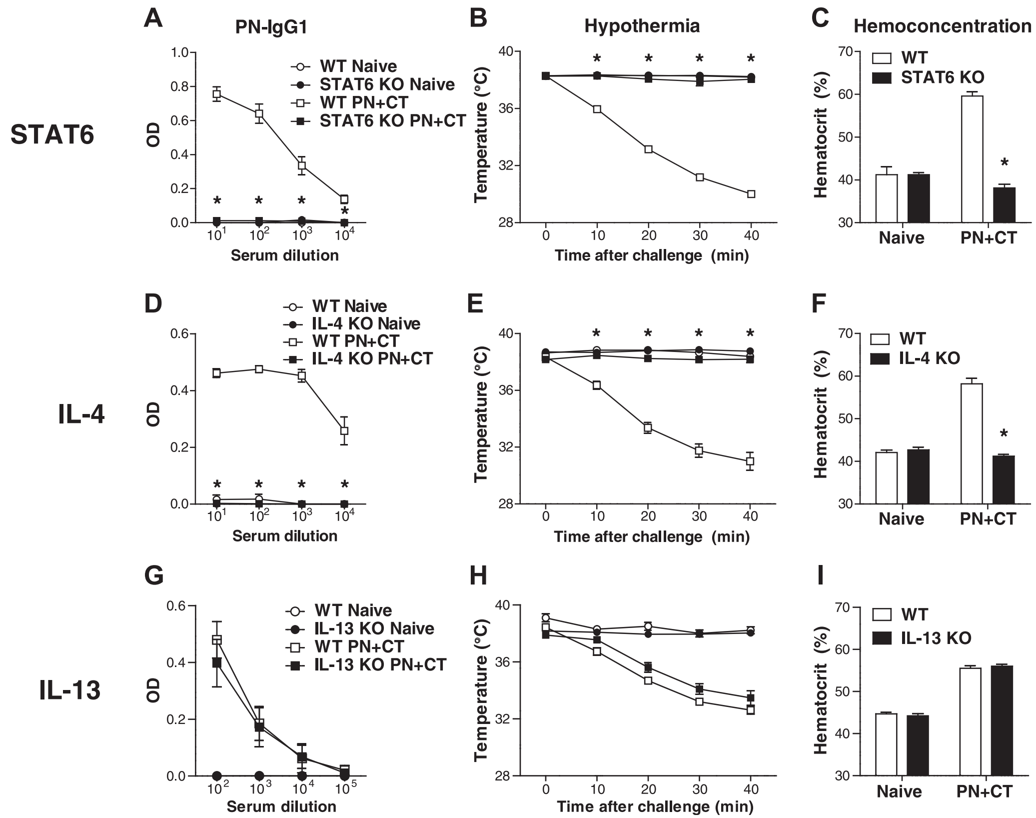

FIG E3. Peanut *(PN)* sensitization follows canonical T_H2 dependency on STAT6 and IL-4. WT **(A-I)**, STAT6 KO (Fig E3, *A-C*), IL-4 KO (Fig E3, *D-F*), or IL-13 KO (Fig E3, *G-O*) mice received peanut plus CT intragastrically weekly for 4 weeks and were challenged with peanut intraperitoneally 1 week later. Fig E3, *A*, *D*, and *G*, Serum peanut-specific IgG_1 levels 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E3, *B*, *E*, and *H*) and hematocrit levels (Fig E3, *C*, *F*, and *I*). Shown are 2 experiments with 3 to 8 mice per group per experiment. *$P < .05$ versus WT peanut plus CT.

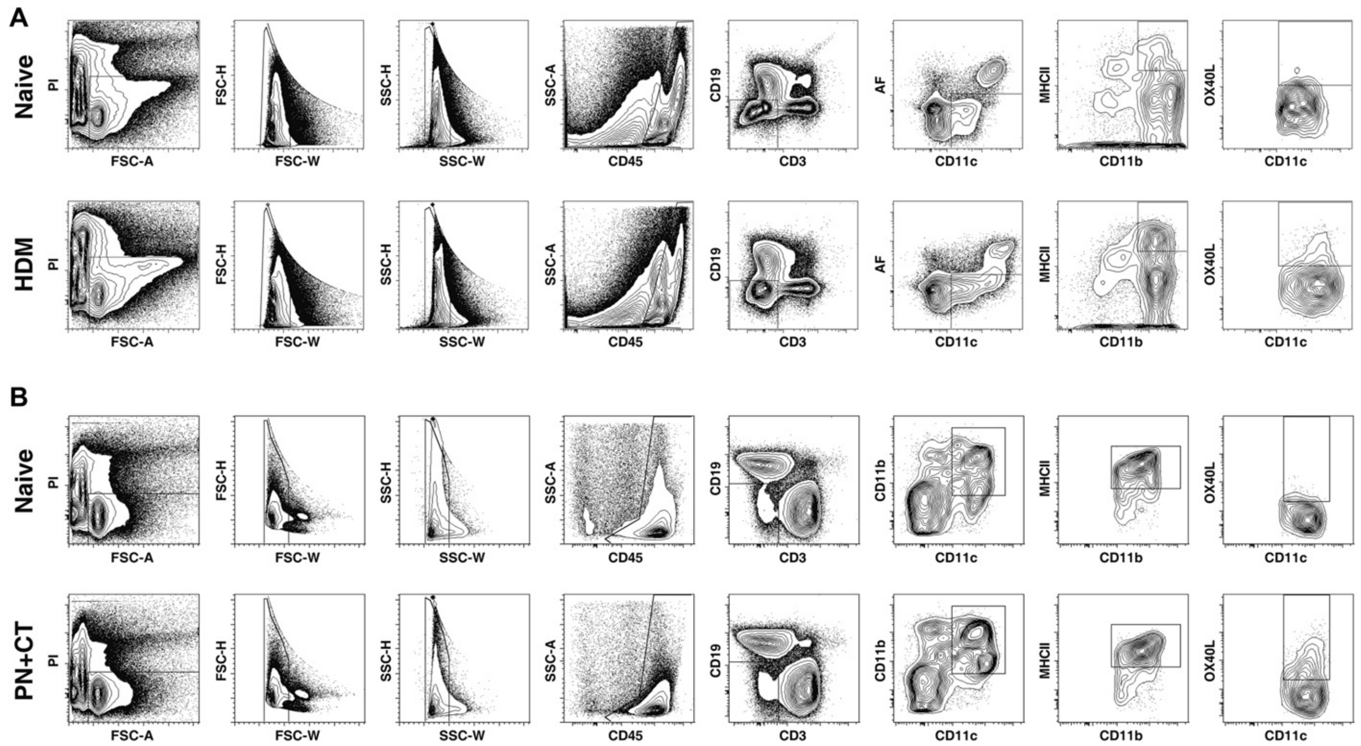

FIG E4. Myeloid DCs expand after 3 days of HDM or peanut *(PN)* plus CT exposure. Flow cytometric gating strategy for myeloid DCs in the lung **(A)** or mesenteric LNs **(B)**. Representative plots are from 2 to 3 experiments. *AF*, Autofluorescence; *FSC*, forward scatter; *PI*, propidium iodide; *SSC*, side scatter.

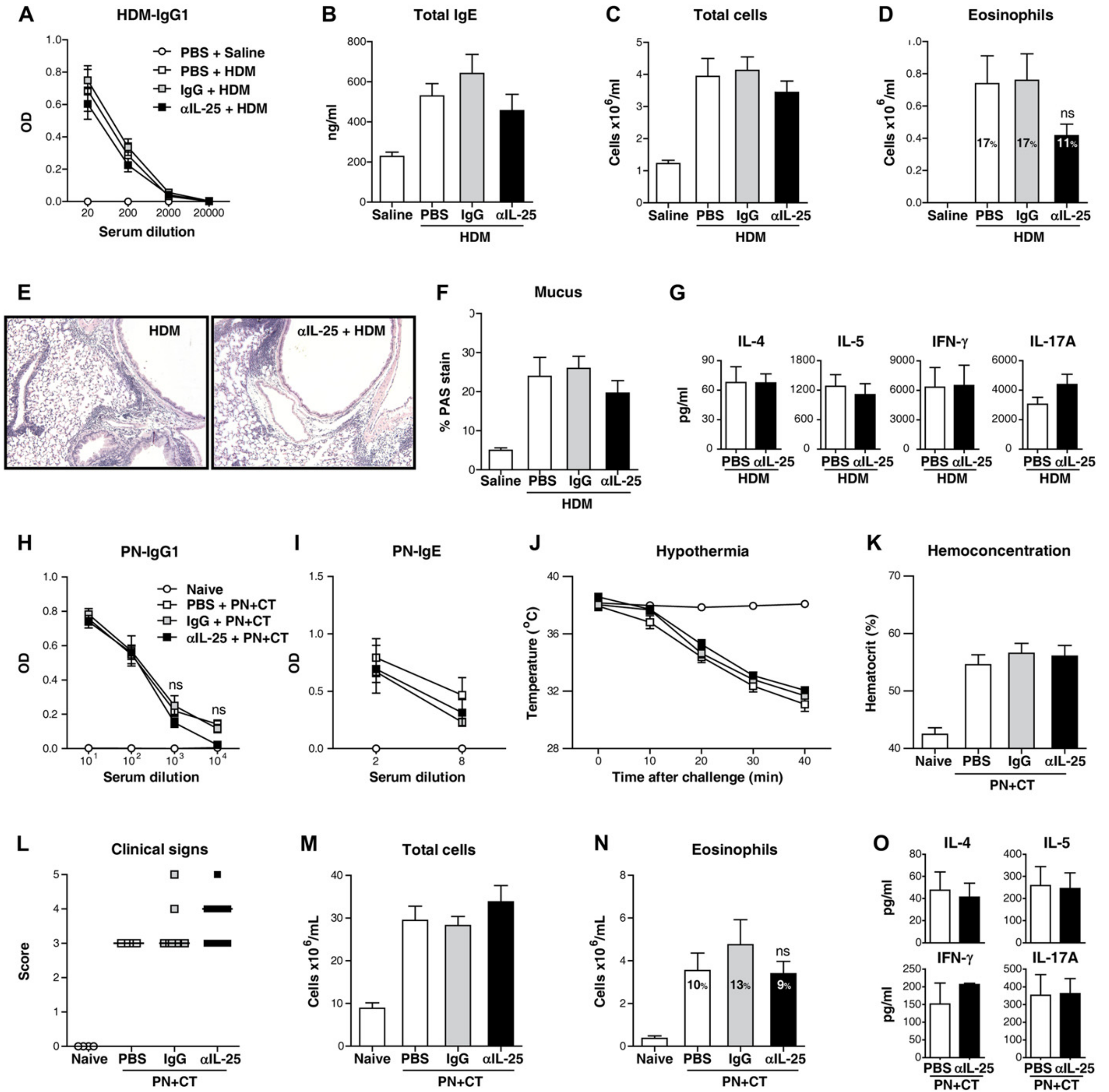

FIG E5. IL-25 neutralization does not significantly affect pulmonary and gastrointestinal allergic sensitization to HDM and peanut *(PN)*. **A-G**, WT mice received PBS vehicle, rat IgG, or 1 mg of anti–IL-25 neutralizing antibody 1 day before and throughout 10 days of HDM exposure. Serum HDM-specific IgG_1 (Fig E5, *A*) and total IgE (Fig E5, *B*) levels. Total cell (Fig E5, *C*) and eosinophil (Fig E5, *D*) counts in bronchoalveolar lavage fluid; mean percentages of eosinophils are shown in *bars*. Fig E5, *E*, Representative lung sections stained with hematoxylin and eosin (×10 magnification) or periodic acid–Schiff *(PAS)* insets (×40 magnification) to assess leukocyte infiltration and mucus production. Fig E5, *F*, Morphometric analysis of periodic acid–Schiff staining. Fig E5, *G*, Cytokine production from HDM-stimulated splenocytes. **H-O**, WT mice received rat IgG or anti–IL-25 antibody 1 day before peanut plus CT intragastrically weekly for 4 weeks and then were challenged with peanut intraperitoneally 1 week later. Serum peanut-specific IgG_1 (Fig E5, *H*) and IgE (Fig E5, *I*) levels 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E5, *J*), hematocrit levels (Fig E5, *K*), and clinical signs (Fig E5, *L*). Total (Fig E5, *M*) and eosinophil (Fig E5, *N*) cell counts in peritoneal lavage; mean percentages of eosinophils are shown in *bars*. Fig E5, *O*, Cytokine production from peanut-stimulated splenocytes. Shown are 2 to 3 independent experiments with 3 to 6 mice per group per experiment. *ns*, Not significantly different.

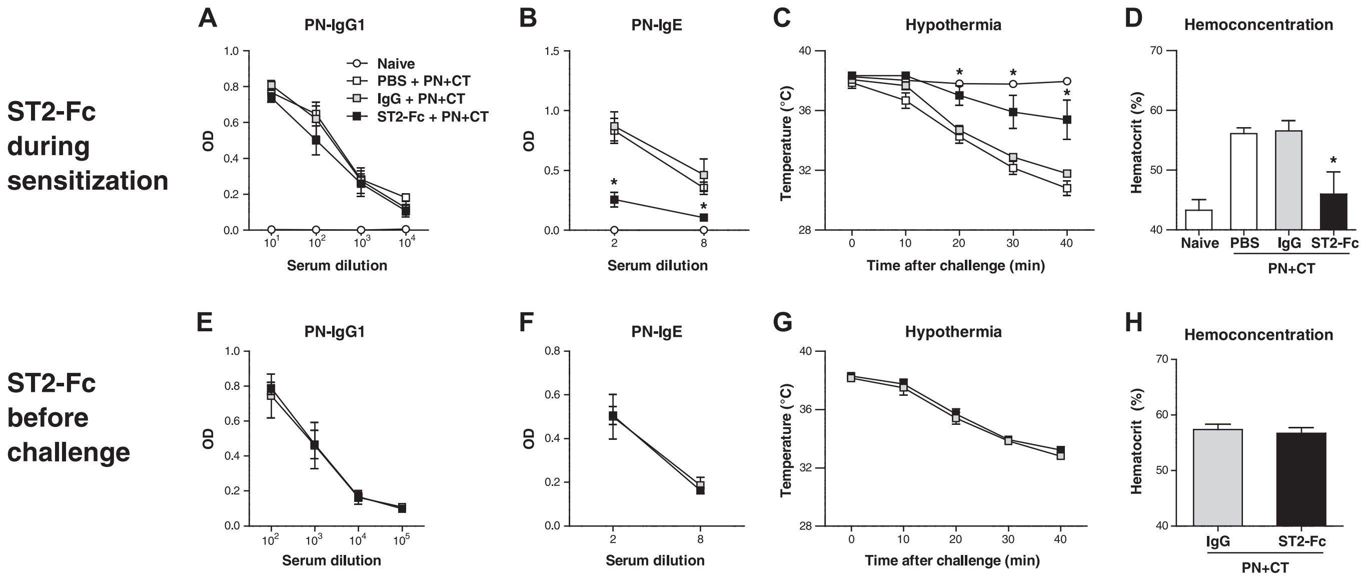

FIG E6. Differential effect of ST2-Fc treatment on peanut *(PN)* sensitization versus challenge. WT mice received PBS vehicle, human IgG, or ST2-Fc 1 day before each sensitization **(A-D)** or challenge **(E-H)**. Serum peanut-specific IgG_1 (Fig E6, *A* and *E*) and IgE (Fig E6, *B* and *F*) levels 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E6, *C* and *G*) and hematocrit levels (Fig E6, *D* and *H*). Shown are 2 independent experiments with 3 to 8 mice per group per experiment. **P* < .05 versus control PBS- or IgG-treated mice exposed to peanut plus CT.

Chapter 4.

A GM-CSF/IL-33 Pathway Facilitates Allergic Airway Responses to Sub-Threshold House Dust Mite Exposure

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FULL TITLE

A GM-CSF/IL-33 pathway facilitates allergic airway responses to sub-threshold house dust mite exposure

RUNNING TITLE: GM-CSF increases HDM susceptibility via IL-33

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ABSTRACT

Allergic asthma is a chronic immune-inflammatory disease of the airways. Despite aeroallergen exposure being universal, allergic asthma affects only a fraction of individuals. This is likely related, at least in part, to the extent of allergen exposure. Regarding house dust mite (HDM), we previously identified the threshold required to elicit allergic responses in BALB/c mice. Here, we investigated the impact of an initial immune perturbation on the response to sub-threshold HDM exposure. We show that transient GM-CSF expression in the lung facilitated robust eosinophilic inflammation, long-lasting antigen-specific Th2 responses, mucus production and airway hyperresponsiveness. This was associated with increased IL-33 levels and activated CD11b$^+$ DCs expressing OX40L. GM-CSF-driven allergic responses were significantly blunted in IL-33-deficient mice. IL-33 was localized on alveolar type II cells and *in vitro* stimulation of human epithelial cells with GM-CSF enhanced intracellular IL-33 independently of IL-1α. Likewise, GM-CSF administration *in vivo* resulted in increased levels of IL-33 but not IL-1α. These findings suggest that exposures to environmental agents associated with GM-CSF production, including airway infections and pollutants, may decrease the threshold of allergen responsiveness and, hence, increase the susceptibility to develop allergic asthma through a GM-CSF/IL-33/OX40L pathway.

KEYWORDS: Allergy; Allergic asthma; Sensitization; Inflammation; House Dust Mite; Th2 Immunity; GM-CSF; IL-33; ST2; OX40L; IL-1α; CD11b$^+$ Dendritic Cells; Alveolar Type II Cells; Threshold; Mouse; NHBE Cells; A549 Cell Line.

INTRODUCTION

Allergic asthma is a complex immune-driven disease of the airways that may develop in susceptible individuals in response to aeroallergen exposure. While allergen exposure is universal, the prevalence of allergic sensitization and asthma is 35% and 10%, respectively [1,2]. A number of reasons may contribute to explain why the relationship between exposure, sensitization and disease is not linear. As allergic asthma occurs in susceptible individuals, many studies have investigated genetic predisposition, or inheritable susceptibility. In this regard, human polymorphisms in over a hundred genes with very diverse biological functions have been associated with atopy and asthma. However, genetic association studies exhibit poor reproducibility in different populations, and statistical correlations provide a weak evidence of causality [3]. The dramatic increase in the prevalence of asthma over the last five decades in Western countries [2] intimates the contribution of factors other than genetic predisposition to the development of asthma. Given that allergen exposure often occurs concurrently with exposures to biologicals (*e.g.* viruses, bacteria) and/or chemicals (*e.g.* pollution), we argued that environmental exposures affecting the immune status of the lung could lower the threshold of allergen responsiveness and precipitate the onset of the allergic diathesis.

With respect to HDM, the most pervasive indoor aeroallergen worldwide, we previously provided a comprehensive computational view of the impact of dose and length of exposure on allergic responses [4]. Importantly, this analysis precisely identified system parameters such as distinct thresholds for different inflammatory and immunological variables. Here, we set out to investigate *whether* and *how* a change in the immune status of the lung at the time of incipient allergen exposure would impact subsequent immunological and physiological responses. As a defining characteristic of complex systems is their sensitivity to initial conditions [5], we elected to

introduce a perturbation to transiently influence key immunological events, namely the state of the antigen-presenting cell (APC) compartment. Using an adenoviral vector approach, we overexpressed GM-CSF in the airway only during early exposure to HDM. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a powerful natural cytokine able to stimulate the proliferation, maturation and function of APCs [6]. As it is produced in response to a variety of environmental stimuli [7-12], it may be viewed as a pervasive immune activator.

Our data show that transient expression of physiologically relevant levels of GM-CSF in the lung lowers the threshold of allergen required to generate allergic airway inflammation and dysfunction by at least ten times. This is associated with a marked increase in the number of activated $CD11b^+$ dendritic cells (DCs) in the lung. Mechanistically, we show for the first time that GM-CSF significantly increases IL-33 expression by alveolar type II cells (ATII), and that GM-CSF-driven responses, including lung eosinophilia, Th2 cytokine production and expression of the critical co-stimulatory molecule OX40 ligand (OX40L), are substantially blunted in IL-33-deficient mice. Collectively, these findings suggest that a wide array of environmental exposures, especially at the time of initial contact with aeroallergens, may increase the susceptibility to develop allergic asthma through a GM-CSF/IL-33/OX40L pathway.

RESULTS

GM-CSF facilitates and exacerbates inflammatory responses to HDM

We have previously reported that daily exposure to 0.2, 1, 5 and 25 μg of HDM induces absent, incipient, moderate and severe immune-inflammatory responses, respectively [4]. Here, we sought to examine the effect of GM-CSF on the responsiveness to HDM. In order to overexpress GM-CSF in the lung, mice were instilled intranasally with $3x10^7$ plaque-forming units (pfu) of a replication-deficient adenovirus encoding a mouse GM-CSF transgene (Ad/GM-CSF). Consistent with our previous reports [13,14], GM-CSF expression peaked in the bronchoalveolar lavage fluid (BAL, ~90 pg/ml) and lung homogenates (~50 pg/ml) at 3 to 7 days after Ad/GM-CSF delivery and sharply decreased by 2 weeks, remaining very low and comparable to the levels detected in the empty Ad (Ad/-) and PBS treated groups (**Figure 1a** and data not shown). GM-CSF was not detected in peripheral blood (not shown). Hence, this adenoviral vector approach leads to transient and compartmentalized GM-CSF release in the lung.

Allergic airway inflammation was evaluated after 2 weeks of intranasal HDM exposure, a time-point at which inflammation reaches the maximal level [4]. Here, Ad/GM-CSF, Ad/- or PBS were given 24 h before daily exposure to saline or increasing doses of HDM (schematic in **Figure 1b**). As shown in **Figure 1c**, HDM alone elicited airway inflammation in a dose-dependent manner with increased number of inflammatory cells starting at 1-5 μg HDM and reaching a plateau at 25 μg, in agreement with our previous studies [4]. In the presence of GM-CSF, inflammation was significantly increased across all HDM doses, including an almost 4-fold increase in mice exposed to 0.2 μg HDM. It is important to note that 0.2 μg HDM is a sub-threshold dose that does not induce any detectable immune-inflammatory response *per se*. In addition, five times less HDM (*i.e.* 5 μg) was required to elicit maximal responses in mice

expressing GM-CSF. The best fit curves in **Figure 1d** clearly show increased sensitivity (left shift), reactivity (greater slope) and maximal inflammatory responses in Ad/GM-CSF versus PBS-treated mice. These increases were mainly due to airway eosinophils (**Figure 1e,f**) and mononuclear cells (**Figure 1g**). Similarly, lung eosinophils ($CD45^{+}SiglecF^{+}CD11b^{+}CD11c^{-}$) were significantly elevated in mice exposed to Ad/GM-CSF along with 0.2 μg HDM (heretofore referred as Ad/GM-CSF+0.2) (**Figure 1h**). In all groups receiving Ad/GM-CSF, neutrophils were slightly increased (<5%) as compared to the PBS controls (~1%, not shown). Tissue inflammation (**Figure 1i**) paralleled BAL data. Peribronchial and perivascular inflammation was clearly observed in mice exposed to Ad/GM-CSF+0.2 but absent in the HDM alone group. We also examined goblet cell hyperplasia and hypertrophy in PAS-stained lung sections (**Figure 1j**). A morphometric analysis of the main airway epithelium revealed significantly greater mucus production in mice exposed to Ad/GM-CSF+0.2 compared to PBS+0.2 (**Figure 1k**). Thus, these findings show that GM-CSF overexpression facilitates and synergistically exacerbates inflammatory responses to HDM. From this point forward, we focused our studies on the effects of the sub-threshold dose of HDM.

GM-CSF promotes type 2 immunity to sub-threshold HDM exposure

To examine whether these inflammatory changes involved an adaptive immune response, we evaluated lymphocyte populations in the lung and draining thoracic lymph nodes. **Figure 2a,b** depicts increases in the proportion and number of activated, effector and memory $CD4^{+}$ T cells ($CD45^{+}CD11c^{-}CD3^{+}CD4^{+}$ and $CD69^{+}$, $CD25^{+}$ or $CD44^{+}$, respectively) in Ad/GM-CSF+0.2 exposed mice. Upon stimulation of splenocytes with HDM *in vitro*, Th2 cytokines (IL-5 and IL-

13), IL-10, Th1 (IFN-γ) and TNF-α were significantly elevated in Ad/GM-CSF+0.2-treated mice (**Figure 2c**), confirming an antigen-specific response.

Similarly, the number and proportion of activated B cells ($CD45^+CD3^-CD19^+$ and $CD69^+$ or $CD86^+$) were greater in the presence of Ad/GM-CSF (**Figure 2d,e**). B cell functionality, as assessed by the levels of serum IgE and HDM-specific IgG1, was also significantly elevated (**Figure 2f,g**). Hence, a GM-CSF-enriched environment promotes allergic sensitization in mice receiving a sub-clinical dose of HDM (0.2 μg).

Transient GM-CSF overexpression leads to HDM-specific long-term responses

We next sought to investigate the long-term impact of these early GM-CSF-induced immune-inflammatory changes. To this end, we followed two different experimental protocols. As shown in the diagram in **Figure 3a**, mice received Ad/GM-CSF or PBS and 0.2 μg HDM for 10 consecutive days; then, allergen exposure was discontinued for 4 weeks until inflammation was resolved [15], at which time animals were re-exposed to saline or sub-threshold doses of HDM for 3 consecutive days. Note that such short exposure does not induce any detectable inflammatory response in naïve mice (not shown). As depicted in **Figure 3b,d**, re-exposure with 0.2 μg HDM resulted in robust inflammation, rich in eosinophils, only in mice that initially received Ad/GM-CSF+0.2. The levels of HDM-specific IgE and IgG1 were also significantly elevated (**Figure 3e,f**). Re-exposure of sensitized mice to a ten times lower dose of HDM (0.02 μg) already induced a mild inflammatory response with 4.3% eosinophils (not shown). As expected, animals initially receiving PBS+0.2 were not sensitized and, therefore, did not respond to subsequent HDM re-exposure. Thus, initial GM-CSF overexpression induces an antigen-

specific immune response that maintains memory capacity resulting in robust immune-inflammatory responses after exposure to sub-threshold doses of allergen.

We, then, used an extended protocol of allergen exposure (daily HDM for 4 weeks) to evaluate lung function. Only mice that received Ad/GM-CSF+0.2 exhibited greater airway hyperresponsiveness. In particular, airway (Rn, **Figure 3g**) and tissue (G, **Figure 3h**) resistance to nebulized methacholine (MCh) were significantly higher, reflecting greater bronchoconstriction and heterogeneity of small airway narrowing. These findings suggest that an early and transient perturbation of the immune system can have a long-lasting immunological and physiological impact after exposure to sub-clinical allergen doses.

GM-CSF increases antigen presentation

We next examined the surveillance status of the lung by flow cytometric analysis (**Figure 4a**) at day 7, *i.e.* at a time when GM-CSF levels are elevated. GM-CSF significantly increased the proportion (**Figure 4b**) and numbers (not shown) of DCs ($CD45^+CD11c^+MHCII^{hi}B220^-Gr1^-$), and Ad/GM-CSF+0.2 resulted in a greater change. Within this DC population, approximately 80-90% of cells expressed CD11b. In particular, GM-CSF overexpression increased the number of total and activated $CD11b^+$ DCs (**Figure 4c,d**) in the lung, as assessed by expression of MHC class II and the co-stimulatory molecule CD86. As shown in **Figure 4c**, mice exposed to Ad/GM-CSF+0.2 had over ten times more activated $CD11b^+$ DCs. Hence, GM-CSF overexpression changes the nature and activation status of APCs in the lung, arguably facilitating responsiveness to sub-threshold amounts of HDM.

GM-CSF-induced allergic responses to HDM require IL-33

We have recently reported that exposure to 25 µg HDM, a dose that elicits maximal allergic responses, upregulates IL-33, and that IL-33 signalling is required for allergic sensitization in part via upregulation of the co-stimulatory molecule OX40L on DCs [16]. Here, we quantified IL-33 protein in lung homogenates and observed only baseline levels in mice receiving 0.2 µg HDM (**Figure 5a**). Administration of Ad/GM-CSF and Ad/GM-CSF+0.2 increased the levels of IL-33 in the lung. IL-33 was not detected in the BAL (not shown). We next studied the relevance of IL-33 in this GM-CSF-driven model. As depicted in **Figure 5b**, airway inflammation upon Ad/GM-CSF+0.2 delivery was significantly decreased in IL-33 KO mice compared to WT controls. In particular, IL-33 KO mice had over 60% less eosinophils (**Figure 5c**) and IgE (**Figure 5d**), indicating a marked dampening of Th2 allergic responses. In addition, HDM-stimulated splenocytes from sensitized IL-33 KO mice produced significantly less IL-4 (**Figure 5e**), a critical Th2 cytokine in HDM allergy [17]. Lastly, we assessed the expression of OX40L on $CD11b^+$ DCs in the lung (**Figure 5f,g**). The proportion and numbers (not shown) of $OX40L^+$ DCs were clearly elevated in WT mice exposed to Ad/GM-CSF+0.2 but not in IL-33 KO mice, which had comparable levels to the PBS+saline controls.

Similar results were observed in BALB/c ST2 KO mice (see **Supplementary Figure 1A-C** online); ST2 being the IL-33 receptor [18]. Collectively, these data show that GM-CSF-induced allergic sensitization and airway inflammation require IL-33 signaling.

GM-CSF induces IL-33 from alveolar type II cells

Recent reports have suggested that alveolar macrophages are important sources of IL-33 in the lung upon helminth and viral infections [19,20]. At variance with these observations, we found

that IL-33 protein levels from alveolar macrophages stimulated with either HDM, recombinant GM-CSF (rGM-CSF) or LPS *in vitro* were below the limit of detection in both supernatants and cell lysates (**Figure 6a**). These results were consistent at different time-points and at a range of doses (not shown). In contrast, alveolar macrophages stimulated under these conditions produced significantly increased IL-6 (**Figure 6a**) and TNF-α (see **Supplementary Figure 1D** online). The ability of macrophages to upregulate IL-33 seems to be site-specific since peritoneal macrophages from naïve mice stimulated under identical conditions, including rGM-CSF, showed increased intracellular IL-33 (**Figure 6b**). IL-33 was again undetected in supernatants (not shown).

IL-33 expression has been reported in lung epithelial cells and vascular endothelial cells of both mice and humans [18,21-23]. Thus, we performed an immunohistochemistry analysis of lung sections with anti-IL-33 Ab. Images in **Figure 6c** show positive and specific IL-33 staining of epithelial cells located in the alveoli but not conducting airways, inflammatory aggregates or vasculature. In agreement with the above *in vitro* data, alveolar macrophages did not stain for IL-33 (high-magnification image in **Figure 6c**). IL-33^{+} cells were cuboidal and located at alveolar-septal junctions, suggesting these cells are airway epithelial type II cells, also known as type II pneumocytes or alveolar type II cells (ATII). As ATII cells specifically produce surfactant protein C (SPC) [24], we confirmed their identity by staining adjacent lung tissue sections with anti-pro-SPC Ab; indeed, all IL-33^{+} cells co-localized with pro-SPC^{+} cells (**Figure 6d,e**). IL-33 was highly localized in the nucleus of ATII cells, and there was no evidence of cytoplasmic or extracellular localization. As expected, control slides demonstrated no IL-33 (**Figure 6d**, top right image) or pro-SPC (**Figure 6d**, bottom right image) staining. A quantitative analysis revealed a rare, scattered presence of weakly IL-33^{+} cells in lungs from mice exposed to saline,

Ad/- or 0.2 μg HDM alone (**Figure 6f,g**). In contrast, there were significantly more IL-33$^+$ cells with more intense nuclear immunostaining in Ad/GM-CSF and Ad/GM-CSF+0.2 exposed mice (**Figure 6f,g**). IL-33$^+$ cells were primarily located in the upper and middle respiratory tract, and were rare in the lower lung (not shown), which parallels the regions of Ad infection and subsequent expression of GM-CSF. Thus, these data show that GM-CSF increases the number of IL-33-expressing ATII cells as well as the amount of nuclear IL-33 on a *per* cell basis.

GM-CSF modulates IL-33 expression independently of IL-1α

To determine whether GM-CSF could directly upregulate IL-33 in lung epithelial cells, *in vitro* assays were performed using the human ATII cell-derived cell line A549 and normal human bronchial/tracheal epithelial (NHBE) cells. **Figure 7a,b** shows that intracellular IL-33 levels were increased upon stimulation with rGM-CSF. For comparison, cells were exposed to IFN-γ+TNF-α, as these cytokines have been shown to induce IL-33 expression in a variety of cell types [25,26]. Willart *et al.* recently proposed that EC-derived IL-1α can act, in an autocrine manner, to induce GM-CSF and IL-33 production and release [27]. Here, we found that whereas IL-1α was able to upregulate intracellular IL-33 in A549 and NHBE cell cultures, the levels of IL-33 induced by either rGM-CSF or IFN-γ+TNF-α stimulation were not affected by blockade of IL-1α using anti-IL-1α Ab. In addition, we found that *in vivo* administration of rGM-CSF readily elevated IL-33 in the lung while IL-1α levels remained comparable to those in saline-exposed mice (**Figure 7c**). Lastly, we exposed separate groups of mice to either a very high dose (100 μg) or a sub-threshold dose (0.2 μg) of HDM *in vivo* and observed that only exposure to 100 μg HDM significantly increased IL-1α as well as IL-33 in the lung. Thus, these *in vivo* and *in vitro* results demonstrate

that GM-CSF can increase intracellular IL-33 expression from lung epithelial cells, likely in an IL-1α independent manner.

DISCUSSION

HDM has inherent immunogenic properties. The amount of inhaled HDM required to induce sensitization and, eventually, allergic disease in humans remains to be elucidated. However, it is likely that most individuals are exposed to sub-threshold concentrations. As allergen exposure rarely occurs in isolation, understanding the impact of other environmental exposures on allergen responsiveness may shed light on the molecular requirements to develop allergic asthma. Here, we established a system to investigate whether and how environmental triggers leading to GM-CSF production in the lung facilitate the emergence of an allergic asthmatic phenotype upon exposure to, particularly, sub-clinical concentrations of HDM.

We used GM-CSF as a surrogate environmental trigger for several reasons. First, it is a cytokine produced in the lung in response to a variety of environmental stimuli, including bacterial and viral infections, allergens, fungi, cigarette smoke and pollutants [7-12]. Second, GM-CSF is a potent cytokine capable of enhancing differentiation and activation of APCs, particularly DCs [6,13,14], thus influencing incipient responses to allergens. Third, it has been shown that several polymorphisms in the GM-CSF gene are risk factors for the development of atopy and atopic diseases [28,29]. Here, we show that enhanced GM-CSF expression in the airways alters the local environment to promote cardinal immune, inflammatory, structural and functional features of the allergic asthmatic phenotype upon exposure to sub-threshold levels of allergen. We used a replication-defective adenoviral GM-CSF gene transfer approach as a vector system, instead of recombinant cytokines or transgenic mice, to induce extended, but transient, expression of GM-CSF. This approach was previously used in our laboratory to overexpress several cytokines and chemokines, resulting in the induction of distinct immune-inflammatory phenotypes to the same antigen, OVA [13,14,30-32]. The dose of Ad vector used results in levels of

GM-CSF in BAL (~90 pg/ml) that can be considered physiologically relevant [33], does not induce tissue damage and causes only very mild lung inflammation consisting of mononuclear cells but not eosinophils. Importantly, $3x10^7$ pfu of the control viral vector did not cause any detectable immune-inflammatory change in the lung. Thus, for simplicity, we did not systematically include this control throughout this study. Of note, this adenoviral vector system has a remarkable ability to shuttle the GM-CSF transgene to bronchial and alveolar epithelial cells and, to a lesser degree, alveolar macrophages [14].

We have previously reported a comprehensive computational analysis of immune-inflammatory dose-responses to HDM, and shown that continuous exposure to amounts of allergen below the threshold (the threshold being 0.5 µg for HDM-specific IgG1 and 2 µg for eosinophils) does not cause detectable changes in the lung, even after five months [4]. Here, we show that a GM-CSF enriched lung environment increases the sensitivity and reactivity of the host to HDM. Strikingly, about ten times less allergen was needed to trigger allergic responses, including eosinophilic inflammation and goblet cell hyperplasia/hypertrophy with mucus production. These changes were associated with activation of the adaptive immune system, as mice exposed to the sub-threshold dose of 0.2 µg HDM in the context of GM-CSF had significantly more activated Th2 and B cells that produced high levels of IL-5 and IL-13, total IgE, HDM-specific IgG1 and, later, HDM-specific IgE, in accordance with the progression of isotype class switching. We argued that these findings were the consequence of a heightened activation state of the surveillance compartment. In this regard, our data show that GM-CSF alone and, to a greater extent, in combination with 0.2 µg HDM, expanded $CD11c^+MHCII^{hi}$ lung DCs. In addition, most $CD11c^+MHCII^{hi}$ cells were also $CD11b^+$ and exhibited upregulated expression of the co-stimulatory molecule CD86. These cells have been shown to be necessary

and sufficient for the initiation of Th2 responses to HDM [34]; they are rapidly recruited to the lungs during HDM exposure and can migrate to draining lymph nodes [7].

The early immune-inflammatory responses described above were antigen-specific and had long-term consequences, as only mice initially sensitized with Ad/GM-CSF+0.2 exhibited, one month later, eosinophilic inflammation in response to a brief re-exposure with 0.2 µg HDM, and even 0.02 µg HDM. At this time point, high levels of HDM-specific IgG1 and IgE were detected. Lung dysfunction (AHR) was also apparent after extended allergen exposure, with increased airway and tissue resistance to methacholine challenge indicating higher broncho-constriction and heterogeneity of small airway narrowing. Collectively, the data show that incipient expression of GM-CSF in the airway expanded and activated $CD11b^+$ DCs in the lung, thus generating conditions favorable for the induction of an adaptive immune response to otherwise innocuous amounts of HDM.

IL-33 is a novel member of the IL-1 family of cytokines [18]. Higher levels of IL-33 have been detected in the lung of asthmatics [21,25], and genetic analyses have linked polymorphisms in the *il-33* and *st2* genes with allergic disease and asthma [35]. Overexpression of IL-33 in transgenic mice or administration of recombinant IL-33 leads to Th2 responses [16,36,37]. However, discrepant results have been reported in regards to the relevance of IL-33 signaling in promoting allergic responses in ovalbumin-based experimental models [38-41]. We have recently reported that IL-33 plays, along with the co-stimulatory molecule OX40L, a central role in allergic sensitization and inflammation in a HDM-driven system [16]. However, the molecular signals that trigger IL-33 production remain unclear. Here, we show for the first time that GM-CSF increases the levels of IL-33 protein in the lung, and that GM-CSF-driven allergic responses are mediated, at least in part, by IL-33 signalling. Indeed, eosinophilic inflammation, IL-4 production and OX40L

upregulation on DCs were markedly decreased in mice deficient in IL-33 signalling. There is evidence that IL-33 can be produced by a number of cell types under different conditions. For example, bronchial epithelial and vascular endothelial cells have been shown capable of producing IL-33 in both humans and mice [18,21,23]. Alveolar macrophages have been reported to express IL-33 in response to influenza A infection [20] as well as to some, *i.e. Nippostrongylus brasiliensis* [19], but not other, *i.e. Strongyloides venezuelensis* [42], helminth infections. Recent studies have found that ATII cells are the major source of IL-33 at baseline and upon lung inflammation following exposure to OVA, ragweed, papain as well as upon certain fungal and helminth infections [42-45]. We show that GM-CSF expression in the lung led to increased numbers of IL-33$^+$ cells. These cells had morphological features of ATII cells and were also pro-SPC$^+$, a specific ATII cell marker [24]. We did not find IL-33$^+$ alveolar macrophages, nor did they produce IL-33 upon stimulation with rGM-CSF, HDM or LPS *in vitro*. Interestingly, we found that peritoneal macrophages increased intracellular IL-33 upon HDM, rGM-CSF or, as previously published [46], LPS stimulation. These data suggest that GM-CSF can directly activate ATII cells to produce IL-33. From a broader perspective, it seems increasingly clear that the cellular source of IL-33 is dependent on both the site and the context.

Many questions regarding the fundamental biology of IL-33 remain unsolved, in particular the mechanisms of processing and secretion. IL-33 is usually found in the nucleus, where it is thought to act as a nuclear factor, specifically as a histone-binding factor that regulates gene expression [23,47]. In agreement with previous reports [42,43], we were able to detect only nuclear IL-33. However, it is clear that IL-33 can also function extracellularly as a cytokine that activates target cells expressing the ST2 receptor. Indeed, our results in IL-33 KO and ST2 KO mice indicate that IL-33/ST2 interactions drive allergic sensitization to HDM and, thus, IL-33 is

important as a released cytokine. It is likely that rapid degradation after release, by a mechanism yet to be clarified, accounts for the difficulties in measuring IL-33 in fluids.

It has been recently proposed that HDM stimulates epithelial cells to release IL-1α which, then, acts in an autocrine manner to release GM-CSF and IL-33 that mediate DC recruitment and activation [27]. It should be pointed out that these findings were made in a system solely driven by HDM, involving, in some instances, exposure to exceptionally high doses of HDM (100 μg intratracheally). Here, we investigated the involvement of IL-1α in our system. We found that administration of rGM-CSF *in vivo* enhanced IL-33 but not IL-1α levels in the lung. In addition, *in vitro* assays using human A549 and NHBE cells demonstrated that GM-CSF-driven induction of intracellular IL-33 expression is IL-1α independent. Thus, in the likely common real-life scenario of exposure to sub-threshold amounts of allergen, our data propose a novel molecular pathway of Th2 immunity that is independent of IL-1α and where GM-CSF is upstream of IL-33.

In conclusion (**Figure 8**), we have demonstrated that an initial and transient immune perturbation in the lung, namely GM-CSF expression, lowers the threshold of immune responsiveness to HDM and facilitates robust allergic inflammatory responses. We also show that GM-CSF induces intracellular IL-33 expression from ATII cells, and that immune-inflammatory responses to sub-threshold amounts of HDM in the context of GM-CSF expression, including OX40L upregulation, are substantially IL-33 dependent. These data suggest that in a model that is not driven by the allergen (HDM) itself, a GM-CSF/IL-33/OX40L pathway facilitates the development of an allergic asthmatic phenotype. This pathway may be relevant where allergic asthma is precipitated by environmental exposures, such as certain infections and pollution.

METHODS

Animals. Female BALB/c mice (6-8 weeks old) were purchased from Charles River Laboratories and Taconic. IL-33 KO and ST2 KO [40] mice were kindly provided by MedImmune (Gaithersburg, MD) from Taconic (Derwood, MD) and Charles River Laboratories (Frederick, MD), respectively. Mice were housed in a specific pathogen-free environment. All experiments were approved by the Animal Research Ethics Board of McMaster University.

Adenovirus administration. A replication-deficient human type 5 adenoviral construct carrying mouse GM-CSF cDNA in the E1 region of the viral genome (Ad/GM-CSF) was delivered intranasally to isoflurane-anesthetized mice 24 h before the first HDM exposure. Ad/GM-CSF or an E1-deleted control virus (Add170-3 or Ad/-) was administered at a dose of $3x10^7$ pfu in 30 µl PBS, as previously described [13,14].

House dust mite administration. Isoflurane-anesthetized mice received 5-fold increasing doses (0.2, 1, 5 or 25 µg) of HDM (*Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, NC) in 10 µl saline once daily for 7 consecutive days or 5 days/week followed by 2 days of rest for a total of 2 or 4 weeks, as specified. For the *in vivo* recall protocol, mice were exposed to 0.2 µg HDM for 10 consecutive days, then rested for 4 weeks, and re-exposed to saline, 0.02 or 0.2 µg HDM for 3 consecutive days. To evaluate very early events, mice received only one administration of 0.2 or 100 µg HDM, or 4 µg recombinant murine GM-CSF.

Sample collection. Mice were killed 24 h after the last exposure to HDM, unless otherwise specified. **Blood** was collected by retro-orbital bleeding; serum was obtained by centrifugation of whole blood. **Spleens and thoracic lymph nodes** were collected and kept in ice-cold HBSS until processing. The **lungs** were dissected, the trachea was cannulated with a polyethylene tube (BD Biosciences, San Jose, CA) and two lavages (0.25 + 0.2 ml) were done with PBS supplemented

with complete protease inhibitor (Roche, Mannheim, Germany). Total cell numbers were enumerated using a hemocytometer. Cytospins were prepared and stained with Protocol Hema 3 set (Fisher Scientific, Middletown, VA) and ~500 cells were counted and identified as mononuclear cells, neutrophils and eosinophils. Following BAL, the left lobe of the lung was slowly inflated and fixed in 10% formalin for histological analysis. The right lobes were homogenized as previously described [16]. Alternatively, lungs were perfused with PBS and left and/or right lobes were kept in ice-cold HBSS until processing for flow cytometric analysis.

Cell cultures. Splenocytes were isolated and resuspended to $8x10^6$ viable cells/ml in complete RPMI, as previously described [16]. Cells were cultured in triplicate in medium alone or with 15.625 µg/ml HDM in flat-bottom, 96-well plates (BD Biosciences) for 5 days. **Alveolar and peritoneal macrophages** were recovered from the lungs and peritoneal cavity of naïve mice with ice cold PBS supplemented with 0.5 mM EDTA and, then, washed with complete RPMI. 0.5-$1x10^5$ macrophages/well were plated and allowed to adhere at 37 ºC for 1 h. Non-adherent cells were removed by gently washing three times with warm PBS. Macrophages were stimulated with or without increasing doses of HDM (up to 100 µg/ml), recombinant murine GM-CSF (up to 1 µg/ml) or LPS (up to 100 ng/ml; Sigma-Aldrich, St. Louis, MO) for 2, 6, 24, 48 or 72 h. For **A549 cells** (ATCC, Manassas, VA) and **normal human bronchial/tracheal epithelial cells** (NHBE, Lonza, Walkersville, MD), 1-$2x10^5$ cells were allowed to adhere overnight in either Dulbecco's Modified Eagle Medium (DMEM, Gibco-Life Technologies, Grand Island, NY) supplemented with 10% FBS and penicillin/streptomycin, or Bronchial Epithelial Growth Medium BulletKit (Lonza), respectively. The following day, cells were primed with recombinant human IFN-γ (50 ng/ml), TNF-α (10 ng/ml), IL-1α (50 ng/ml) or GM-CSF (50 ng/ml) (all from R&D Systems, Minneapolis, MN) for 24 h with or without neutralizing anti-IL-1α Ab (10 µg/ml;

R&D). Supernatants from both macrophages and epithelial cells were collected and fresh media was added to cells prior to 3-5 cycles of freeze/thawing.

Cell isolation and flow cytometric analysis. Lung cells were isolated by collagenase digestion (Collagenase type I, Gibco-Life Technologies) and passed through 40 μm cell strainers, as previously described [16]. **Lymph nodes** were triturated between frosted slides in HBSS. All cells were washed in FACS buffer (0.5-1% BSA and 10 mM EDTA-supplemented PBS) and filtered again before staining. For each antibody combination, 2-4x10^6 cells were first incubated with anti-CD16/CD32 (eBioscience, San Diego, CA) and then stained for 30 min at 4 ºC. Antibodies were obtained from BD Biosciences, eBioscience, BioLegend (San Diego, CA) or MD Biosciences (Saint Paul, MN) and titrated before use. Propidium iodide (Sigma-Aldrich) and forward scatter/side scatter were used to exclude dead cells and doublets. Data were acquired on a BD LSR II and analyzed with FlowJo software (TreeStar, Ashland, OR). Fluorescence minus one controls were used for gating.

H&E and PAS histology. Paraffin sections of lungs were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS), as previously described [48]. For PAS stain quantification, multiple images (up to 15) of main airways were captured with OpenLab software (version 5.5.0; Improvision) via a MicroPublisher camera (5.0 RTV; QImaging) and Leica microscope (Leica Microsystems). Morphometric analysis was performed using a custom computerized analysis system (Northern Eclipse software version 5; Empix Imaging) that calculates the percentage of tissue area that was positively stained within a 30 μm wide band from the basement membrane extending into the airway lumen.

Immunohistochemistry. Paraffin sections of lungs were deparaffinized in xylene, and hydrated through graded alcohols. To stain for IL-33, heat-induced epitope retrieval was performed using

10 mM citrate buffer and, then, sections were placed in 0.05 M Tris-HCl-Tween buffer. We blocked endogenous peroxidase with 3% H_2O_2 in distilled water, and proteins with 1% BSA. Sections were then incubated with anti-IL-33 Ab (1:50; monoclonal biotinylated Ab, Nessy-1, Enzo Life Sciences, Farmingdale, NY) in Tris+Tween+saponin buffer for 1 h. Finally, we used streptavidin/peroxidase conjugate (Dako, Glostrup, Denmark) and prepared DAB chromogenic substrate. To stain for pro-SPC, endogenous peroxidase was blocked with 3% H_2O_2 in methanol, with HCl added last. Proteinase K (Dako) was used to unmask antigens/epitopes, and proteins were blocked with 5% normal goat serum. Sections were incubated with anti-pro-SPC Ab (1:2000; polyclonal Ab, Millipore, St. Charles, MO)) in Ultra Clean Diluent (Fisher Scientific) for 1 h. Finally, we used EnVision detection system with DAB+ (Dako). Mayer's hematoxylin was used to counterstain. Negative controls were generated by omitting the primary Ab. For enumeration of IL-33$^+$ cells, counts were made from five fields of view *per* mouse (2 from the upper lobe, 2 from the middle and 1 from the lower respiratory tract) at 400x and normalized per unit area (mm^2).

Cytokine and Ig analysis. Murine GM-CSF, IL-33, IL-6, TNF-α and IL-1α as well as human IL-33 were measured by ELISA (DuoSet kits, R&D Systems), and murine IL-4, IL-5, IL-10, IL-13, IFN-γ and TNF-α by Luminex (multiplex kits, Millipore) according to manufacturer's protocols. Total protein content was quantified by using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Total or HDM-specific Ig levels were measured by using ELISA techniques, as previously described [16,49]. HDM-specific Ig levels are depicted as OD – background, where background = average OD of at least 20 blank wells + two standard deviations.

Airway responsiveness. Mice were anesthetized, paralyzed, tracheostomized and mechanically ventilated with a small animal computer-controlled piston ventilator (flexiVent, SCIREQ Inc.).

The response to nebulized saline and increasing doses (3.125, 12.5 and 50 mg/ml) of methacholine (MCh, Sigma-Aldrich) was measured as previously described [4]. Model fits that resulted in a coefficient of determination less than 0.8 were excluded.

Data analysis. Data are expressed as mean ± standard error of the mean and were analyzed and graphed with Prism software version 5 (GraphPad, La Jolla, CA). Shown are either pooled data from 2-3 experiments yielding similar results or 1 representative experiment of 2-4. Statistical analysis was calculated using Student's *t* test (unpaired, two-tailed) and one- or two-way analysis of variance (with Bonferroni *post hoc* test). Differences were considered statistically significant when p values where less than 0.05. For some outcomes, the experimental data was best fitted with the following sigmoidal dose-response function:

$$y = \frac{Bottom + (Top - Bottom)}{\left(1 + \left(\frac{EC50}{x}\right)^{HillSlope}\right)^{order}}$$

where *y* is the response, *x* is the HDM dose, *Bottom* and *Top* are the plateaus (minimum or baseline and maximum *y*), *EC50* is the value of *x* that provokes a response halfway between the *Bottom* and *Top*, and *HillSlope* is the slope factor.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declare no conflict of interest. RK, JSS and AAH are current employees of MedImmune, and AJC is a current employee of Pfizer.

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FIGURE LEGENDS

Figure 1 GM-CSF in the lung facilitates and exacerbates HDM responses. (**a**) GM-CSF by ELISA in BAL and lung homogenates (LH) from naïve mice (time 0) or after Ad/GM-CSF delivery. (**b**) Schematic of the protocol followed in **c-k** (see METHODS). (**c**) Inflammatory cell counts in BAL. (**d**) Best-fit curves for the original data in C. (**e**) Eosinophil numbers (bars) and percentages in BAL, as well as (**f**) best-fit curves. (**g**) Number of mononuclear cells in BAL. (**h**) Proportion of eosinophils ($CD45^{+}SiglecF^{+}CD11b^{+}CD11c^{-}FSC^{low}SSC^{hi}$) in the lung tissue by flow cytometry. Representative lung sections stained with (**i**) hematoxylin and eosin (H&E, x100 magnification) or (**j**) periodic acid–Schiff (PAS, x400, color-inverted). (**k**) Morphometric quantification of PAS staining. N=11-18 mice/group. * $p < 0.05$ in Ad/GM-CSF versus respective PBS group. ND for not determined.

Figure 2 GM-CSF promotes type 2 immunity to HDM. A) Representative flow plots of $CD4^{+}$ T cells ($CD45^{+}CD3^{+}CD19^{-}$) showing expression of CD69 and CD25 in the lung. (**b**) Numbers of total, $CD69^{+}$, $CD25^{+}$ and $CD44^{+}$ $CD4^{+}$ T cells in thoracic lymph nodes. (**c**) Cytokine levels in supernatants of splenocytes stimulated *in vitro* with HDM. (**d**) Proportion of B cells ($CD45^{+}CD3^{-}CD19^{+}$) that are $CD69^{+}$ or $CD86^{+}$ in the lung. (**e**) B cell numbers in the lymph node. Levels of (**f**) total IgE and (**g**) HDM-specific IgG1 in serum. All samples were collected at the end of the 2 week protocol (**Figure 1b**). N=3-4 mice/group in **a,b,d,e** and N=5-15 mice/group in **c,f**. * $p < 0.05$ in Ad/GM-CSF+0.2 versus PBS+0.2 or PBS+25 versus PBS+saline values.

Figure 3 Initial GM-CSF leads to allergic long-term responses. (**a**) Protocol followed in B-F: mice received Ad/GM-CSF or PBS 24 h prior to HDM exposure for 10 d; then, mice were rested for 4 weeks (no allergen) before re-exposure to either saline or HDM for 3 days, with sample

collection occurring 24 h after the last exposure. (**b-d**) Inflammatory cell numbers in BAL. Levels of (**e**) HDM-specific IgE and (**f**) HDM-specific IgG1 in serum. In (**g,h**), mice received Ad/GM-CSF or PBS and either saline or 0.2 μg HDM for 4 consecutive weeks, with 2 days rest in between each week. (**g,h**) Airway hyperresponsiveness based on peak values for Rn and G. N= 4-14 mice/group. * $p < 0.05$ in Ad/GM-CSF+0.2 versus PBS+0.2.

Figure 4 GM-CSF increases DC numbers and activation. (**a**) Gating strategy for DCs. (**b**) Proportion of DCs ($CD45^+CD11c^+MHCII^{hi}$). (**c**) Numbers of activated $CD11b^+$ DCs ($CD45^+CD11c^+CD11b^+MHCII^{hi}CD86^+$). (**d**) Representative flow plots of $CD11b^+$ DCs showing expression of MHCII and CD86. All samples were collected at day 8. N=3-5 mice/group. * $p < 0.05$ in Ad/GM-CSF+saline/HDM versus respective PBS control.

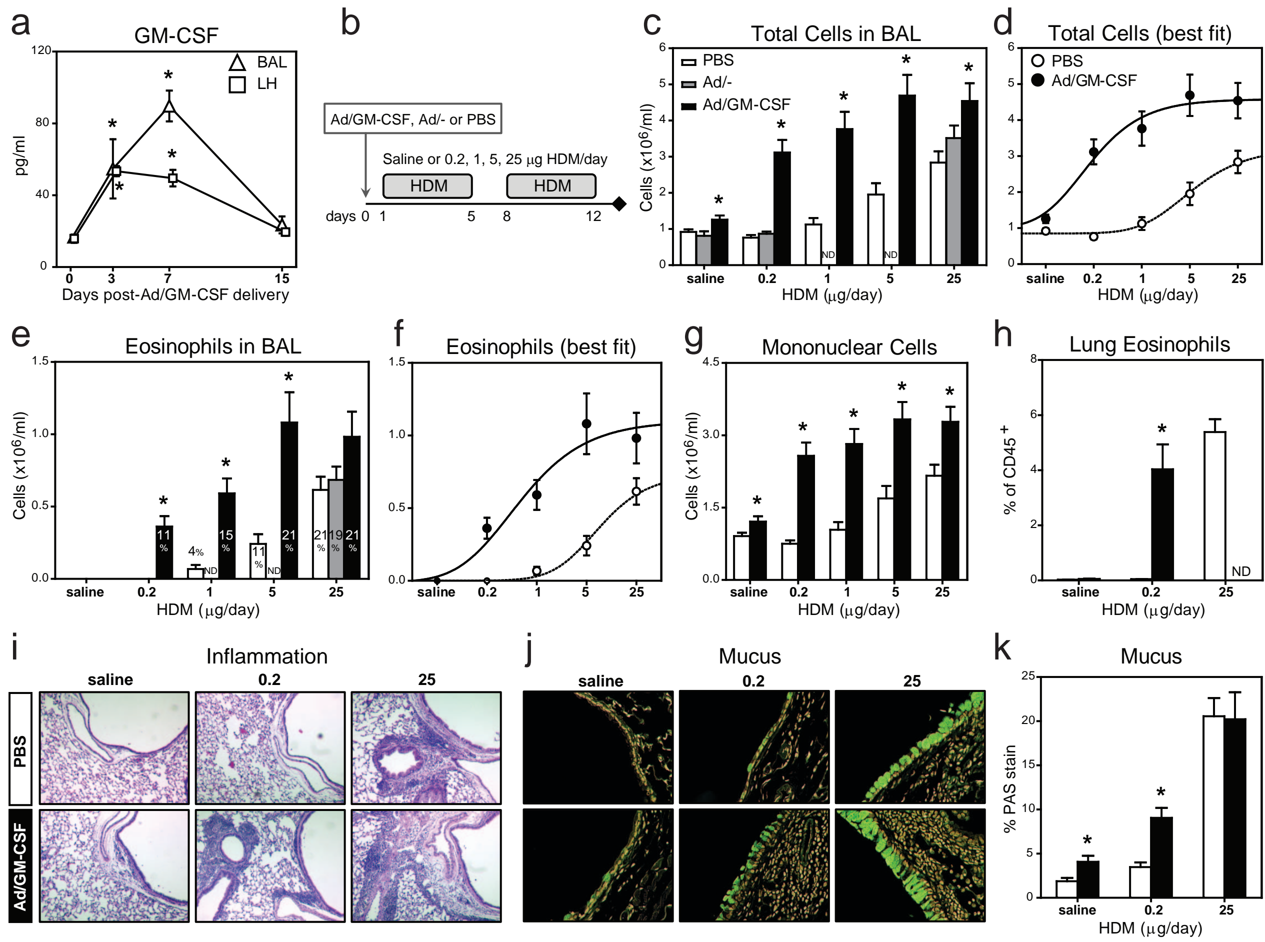
Figure 5 IL-33 mediates GM-CSF-driven responses to HDM. (**a**) IL-33 by ELISA in lung homogenates of WT mice at day 8. BALB/c WT and IL-33 KO mice were exposed to HDM for either (**b-e**) 2 weeks or (**f,g**) 7 consecutive days. (**b**) Total cells and (**c**) eosinophil numbers and percentages in BAL. (**d**) Total IgE in serum. (**e**) IL-4 production by splenocytes stimulated *in vitro* with HDM. (**f**) Proportion of $OX40L^+$ $CD11b^+$ DCs in the lung. (**g**) Representative flow plots showing expression of OX40L on DCs. N=4-19 mice/group. * $p < 0.05$ in (**a**) all groups versus saline, (**b-g**) IL-33 KO versus respective WT.

Figure 6 GM-CSF induces IL-33 on alveolar type II cells and peritoneal, but not alveolar, macrophages. (**a**) IL-33 and IL-6 by ELISA from freeze-thaw alveolar macrophages stimulated *in vitro* for 24 h. (**b**) IL-33 by ELISA from freeze-thaw peritoneal macrophages stimulated *in vitro* for 6, 24 or 48 h. (**c**) Representative lung section upon exposure to Ad/GM-CSF+0.2, stained with anti-IL-33 Ab (nuclear dark brown stain), showing IL-33 immunostaining in alveolar walls

but not in bronchial epithelial cells (BEC), inflammatory cells (INF) or vascular endothelial cells (V) (x200); magnified image (on the right) shows IL-33$^-$ alveolar macrophages (AM) and IL-33$^+$ alveolar type II cells (ATII). (**d**) Representative images (x400) from adjacent lung sections stained with either anti-IL-33 Ab (left image, dark brown in the nucleus) or anti-pro-SPC Ab (middle image, red in the cytoplasm) and negative controls for IL-33 (top right) and pro-SPC (bottom right). (**e**) Enlarged images. (**f**) IL-33$^+$ cell counts in the lung of WT mice (or IL-33 KO as negative control) at 2 weeks. (**g**) Representative images showing distinct intensity of IL-33 stain in Ad/- *versus* Ad/GM-CSF exposed mice. N=6 wells/condition in **a,b** or N=3-7 mice/group in **e**. * $p < 0.05$ versus media or saline.

Figure 7 GM-CSF-driven IL-33 expression is IL-1α independent. (**a,b**) IL-33 by ELISA from freeze/thaw human A549 and NHBE cells stimulated *in vitro* for 24 h with or without neutralizing anti-IL-1α Ab. (**c**) IL-33 and (**d**) IL-1α by ELISA from lung homogenates of WT mice at 18 h after *in vivo* exposure to HDM or rGM-CSF. N=5 mice/group. * $p < 0.05$ versus saline. ND for not determined.

Figure 8 Schematic of the proposed model. Exposure to a variety of environmental stimuli can result in GM-CSF production from bronchial epithelial cells (BEC) and alveolar epithelial type II cells (ATII). GM-CSF induces IL-33 production from ATII cells in an IL-1α-independent manner. Then, IL-33 acts locally on CD11b$^+$ dendritic cells (DC), which reside in close proximity to epithelial cells, to upregulate OX40L and enhance antigen presentation. Such activated status of the lung immunological environment facilitates allergic sensitization to even sub-clinical amounts of inhaled allergens and, ultimately, results in the development of allergic asthma.



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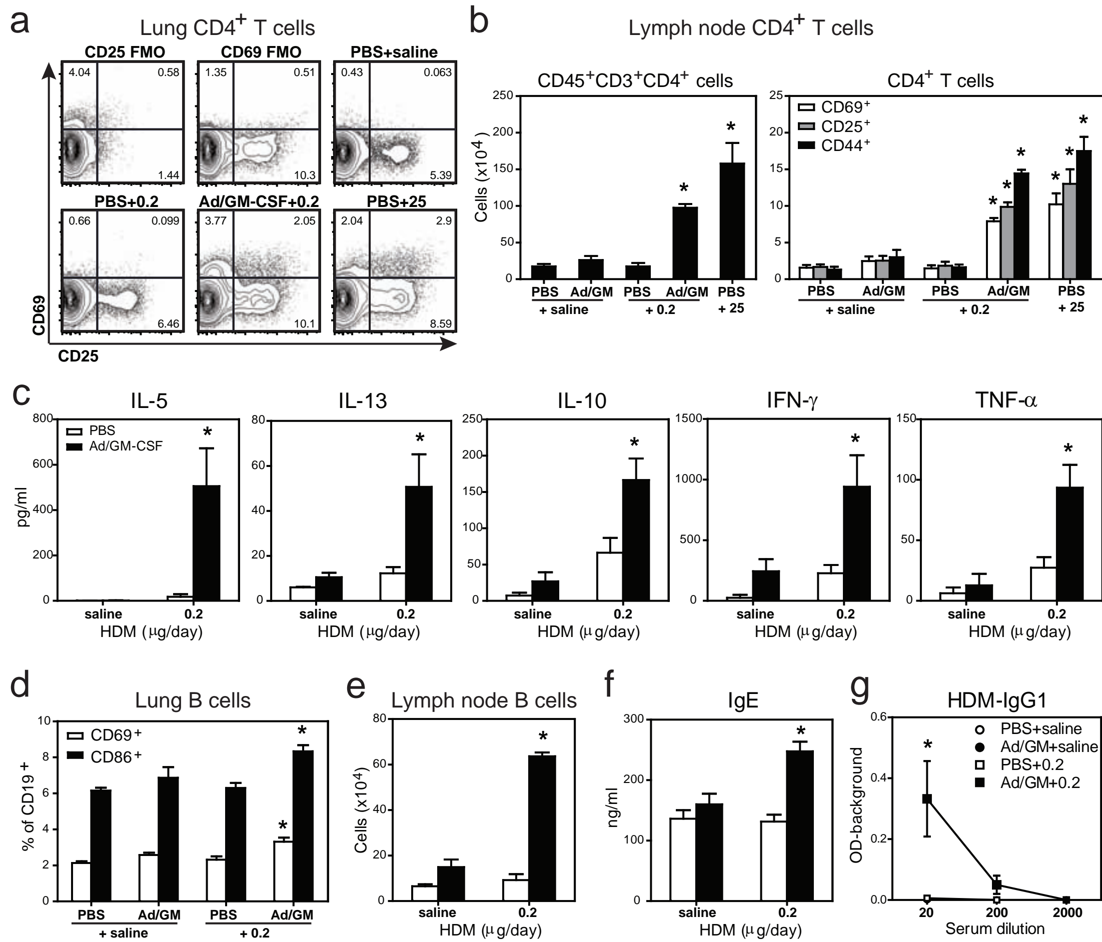
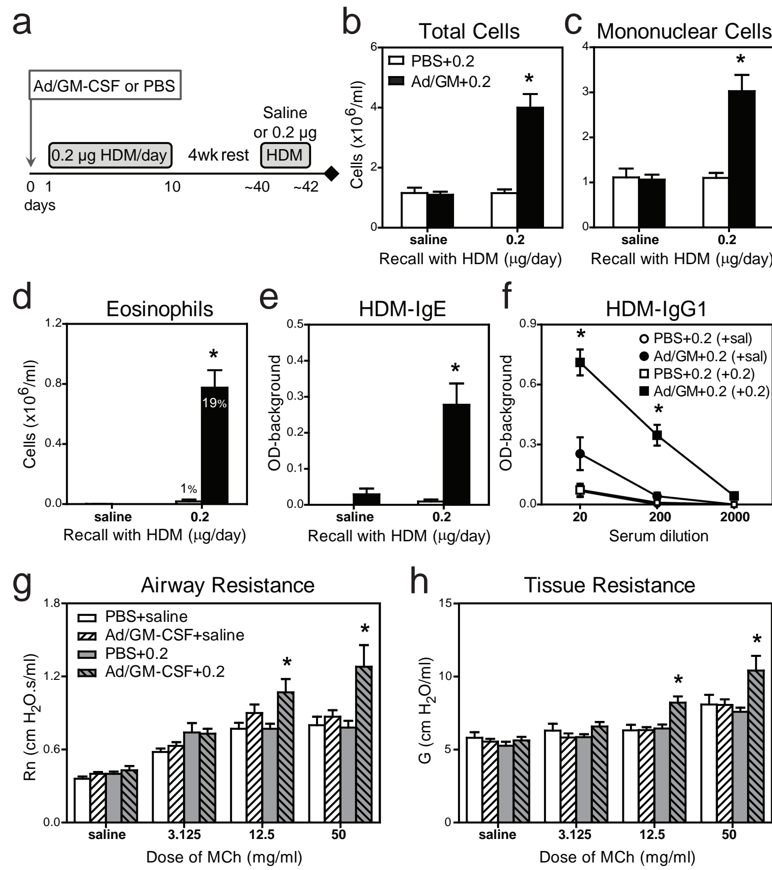


Figure 2.



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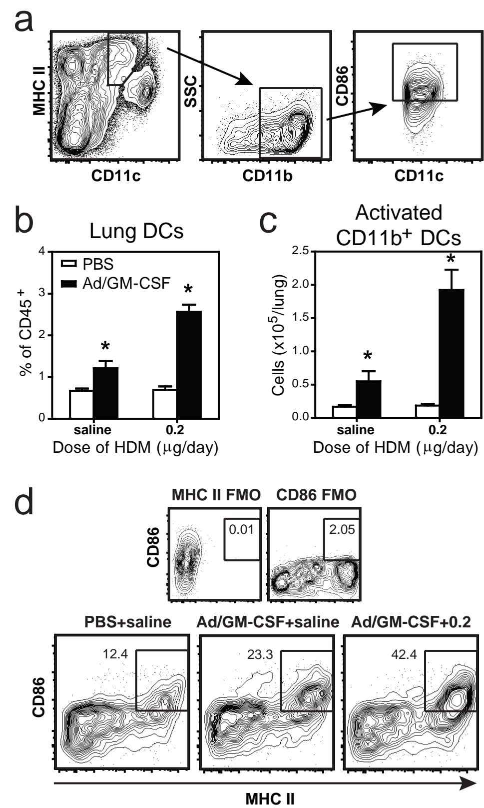

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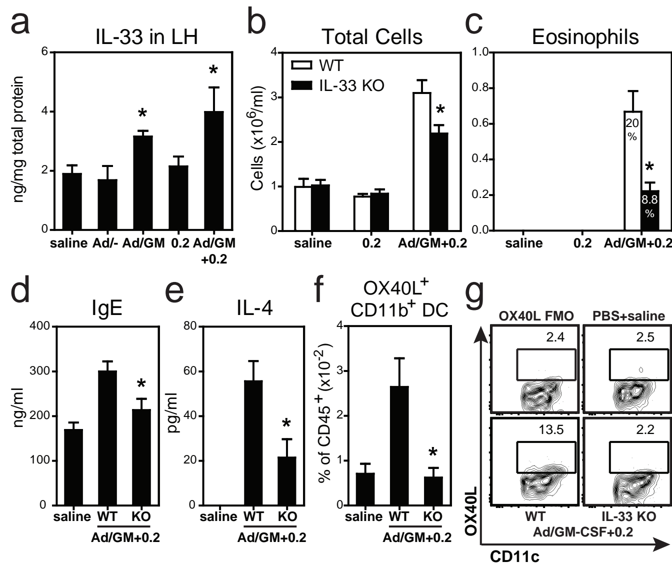

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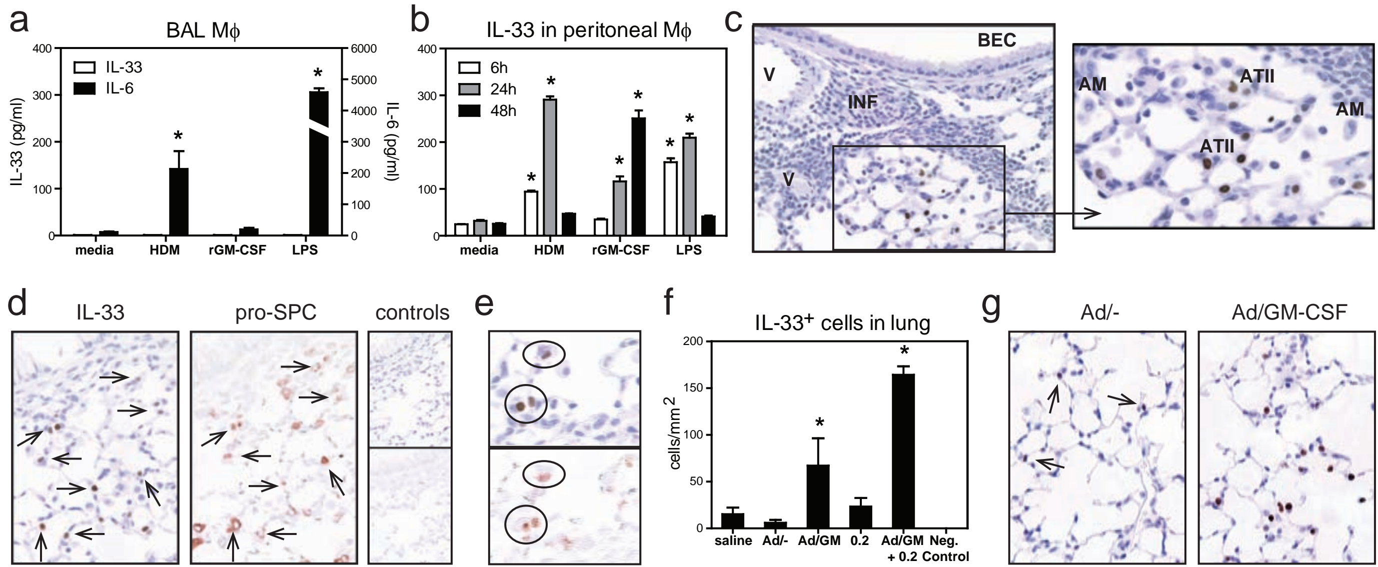

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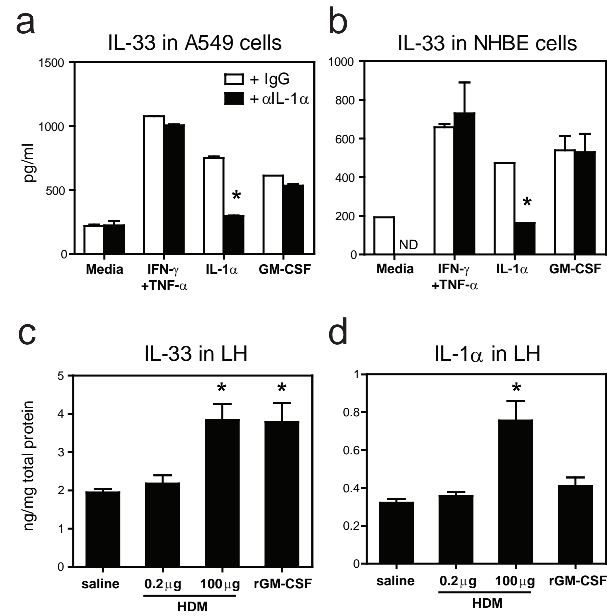


Figure 7.

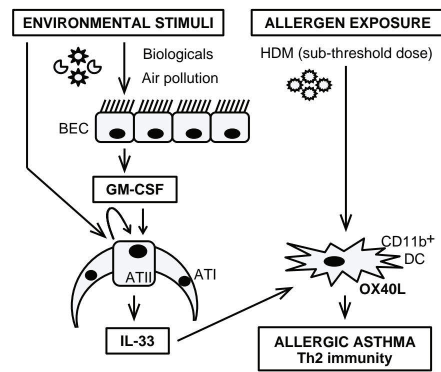
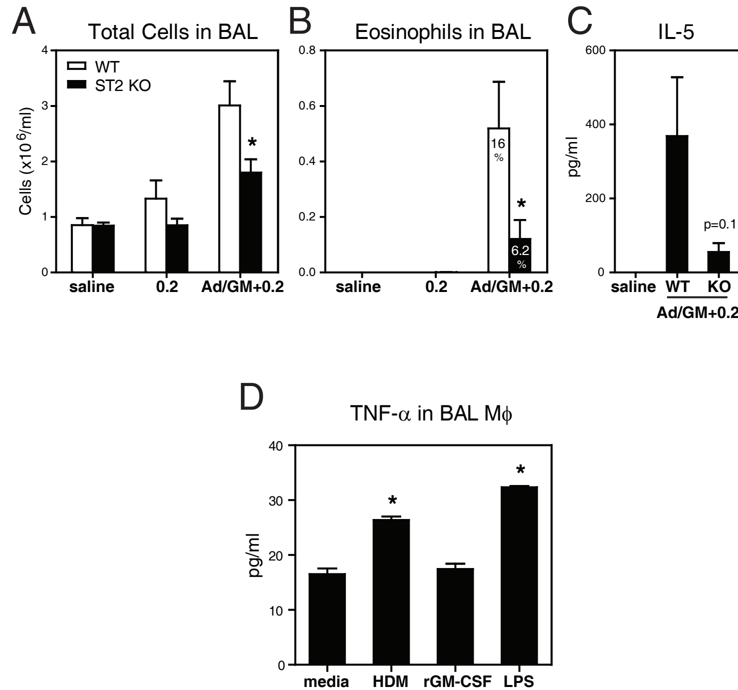


Figure 8.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1 (**A-C**) BALB/c WT and ST2 KO mice were exposed to the 2 wk protocol (as shown in **Figure 1b**). (**A**) Total cells and (**B**) eosinophil numbers and percentages in BAL. (**C**) IL-5 production by splenocytes stimulated *ex vivo* with HDM. For (**A-C**), N=2-9 mice/group and * $p < 0.05$ in ST2 KO versus respective WT. (**D**) TNF-α by ELISA from freeze-thaw alveolar macrophages stimulated *ex vivo* for 24h. N=6 wells/condition and * $p < 0.05$ versus media.

Chapter 5. Discussion

The social and economic burden of allergic asthma is substantial due to its high prevalence, morbidity and mortality both in developed and developing countries. Current therapeutic strategies available for asthma can effectively control most cases of asthma but not cure or prevent the disease. The development of efficient preventative strategies requires a detailed knowledge of the mechanisms underlying the generation of asthma. Dissecting the complex nature of allergic asthma requires, in turn, elucidating the interactions between the immune-respiratory system and the environment, where aeroallergens exist.

The work presented in this thesis focused on studying the ontogeny of allergic asthma. Chapter 2 provides a comprehensive analysis of the impact of allergen exposure on relevant features of the asthmatic phenotype. Chapters 3 and 4 are centered on the investigation of initiating events and critical molecules leading to allergic sensitization and disease expression. We think that, together, these studies advance our understanding of the interactions between the host and the environment, and uncover conditions under which allergic asthma may develop.

Each individual manuscript includes its own discussion. Therefore, the following discussion is intended to extend the interpretation of these collective findings, highlight limitations and outline future directions of this work.

Modeling responses to HDM exposure

Aeroallergens are ubiquitously present in the environment. In particular, HDM is the most pervasive indoor aeroallergen worldwide, and the most common trigger of allergic respiratory reactions. HDM extracts encompass hundreds of protein and non-protein components able to induce an array of responses involving both the innate and adaptive arms of the immune system (59). Our laboratory and, increasingly, others have invested on allergic asthma research in animal models using HDM, instead of the widely used, biochemically simple, surrogate allergen OVA adsorbed to adjuvants. Importantly, while some of the outcomes using OVA and HDM are, under certain conditions, similar, recent evidence has disclosed that the mechanisms underlying these outcomes are different.

Experimental models are often conceived with the goal of establishing a known phenotype. In fact, most research in allergic asthma has been conducted using a specific set of experimental conditions, *i.e.* "static" protocols, such as one allergen concentration at one or a few time points, to achieve a pre-defined outcome. For example, we have typically exposed mice to 25 μg of HDM for either 10 days (68) or several weeks (69) to induce either acute or chronic allergic responses, respectively. While the use of these models has provided novel and useful data, an approach driven by the outcome itself is inherently limited in its ability to discover the underlying conditions to achieve such outcome, and may also produce deceiving outcomes. Indeed, an outcome-driven approach implies that researchers attempt to model what they think is happening, or might happen, rather than what is actually happening. Against this background, it should be pointed out that one of the defining characteristics of living systems is that they are dynamic and constantly reactive; yet, far-reaching conclusions regarding pathogenesis and therapeutics are often drawn from "static" protocols.

In Chapter 2, we aimed at gaining a global understanding of the consequences of allergen exposure on significant immune and physiological parameters using an unbiased and more dynamic approach. Mice were exposed (i.n.) to a range of doses of HDM on a daily basis and evaluations were performed at different time-points for up to a fifth of their lifespan (20 weeks). Then, we generated mathematical equations for each individual biological variable (*i.e*. inflammation and Ig responses) expressed as a function of the dose and length of allergen exposure. To validate the model, we performed several retrodictions as well as predictions (both inter and extrapolations) that were checked against new rounds of experimental data. Lastly, using these equations, we executed *in silico* experiments and used computational tools to predict and visualize how the system would respond to different exposure conditions. This computational analysis revealed non-linear relationships between allergen exposure and allergic sensitization or with airway inflammation. For example, our data show that, within the first 5-7 weeks of allergen exposure, the evolution of total inflammation and eosinophilia in the lung follows a logistic (S shaped)-like pattern with respect to both dose and length of exposure, indicating that responses do not

emerge proportionally to increases in allergen exposure. Similarly, systemic responses, *i.e.* serum levels of HDM-specific Ig, also develop in a logistic manner but following different kinetics, thus suggesting a sequential initiation of allergic events. In humans, several population-based studies have shown a positive association between the extent of HDM exposure and the prevalence of allergic sensitization (137-140). Only Tovey *et al.* found a low risk of sensitization with both low and very high mite exposure, suggesting the possibility of tolerance induction with high doses (141). In fact, this bell-shaped relationship is more common for other allergens, such as animal dander (142). Thus, our data show that the immunologic profile (*i.e.* degree of sensitization and inflammation) quite drastically changes depending on dose and length of exposure to HDM; these multiple outcomes may be mathematically viewed as an illustration of heterogeneity.

Our studies also showed that while total airway inflammation and serum levels of HDM-specific Ig remain elevated over the course of HDM exposure, the eosinophil response dramatically decreases at later time-points. Such a distinct and dynamic behavior intimates the emergence over time of positive and negative signals evolving at different rates; a finding that illustrates biological complexity. Speculatively, it suggests the development of a protective response at later stages to mitigate persistent tissue damage mediated by eosinophils. Whether the decrease in airway eosinophils is due to local regulation, such as increased apoptosis, or modulation at other levels, such as eosinopoiesis or trafficking, requires further investigation.

This computational analysis also uncovered thresholds and points of maximal responsiveness. In particular, maximal inflammatory responses were induced by amounts of HDM in the range of 10 to 15 μg, approximately half the amount that we have typically delivered in studies from our laboratory. Furthermore, distinct threshold doses of allergen for both Ig and eosinophil responses were identified at 0.5 and 2 μg of HDM, respectively. Speculatively, this may contribute to explain the disparity between the prevalence of atopy and asthma in humans. Such detailed information on dose-responses and thresholds in mice was instrumental for the design of future research projects (Chapter 4 and (143)).

A comprehensive understanding of the relationships between allergen exposure, allergic sensitization and allergic disease, which includes inflammation, remodeling and lung dysfunction, may clarify conditions under which allergic asthma may emerge and evolve. This goal could be achieved by developing a global, algorithm-based, model that integrates these variables. In the work described in Chapter 2, we modeled allergic sensitization and inflammation but evaluated lung remodeling (subepithelial collagen deposition) and function (AHR to methacholine) only at one time-point. Therefore, further empirical data on these and other parameters, such as mucus production and smooth muscle mass, would need to be acquired and, then, computationally modeled and incorporated into a more complex model. Such a model would provide qualitative and quantitative knowledge of the relationships among these processes, including the type of relationships and relative contribution of several processes to lung dysfunction, and, ultimately, furnish a novel computational visualization of allergen-host interactions.

Models, whether experimental or mathematical, are inherently limited. That is, there are always more variables that could potentially be included. For example, our *in silico* model is solely based on data using one mouse strain (BALB/c), one sex (females), one age (adults) and one aeroallergen (HDM). While changes in any of these variables may impact the kinetics of the biological processes, it is likely that at least some dynamics would remain similar. For instance, recent studies from our laboratory have shown that neonatal mice are hyporresponsive to HDM (144), *i.e.* higher doses are needed to induce observable responses; yet, responses in neonatal mice follow similar trends (145) to those we have reported in adults. While we could incorporate studies in other strains or age range, we need to consider that the quantity of information that is incorporated into a model may not necessarily impact the quality of the knowledge that can be extracted in return.

The model reported in Chapter 2 is an *empirical model*, *i.e.* a model built on trends provided by experimental data. While we uncovered behaviors, thresholds and relationships between variables, the mathematical equations of the model and the parameters of these equations did not reveal novel immunological properties and processes of the system, allergic asthma. From a mathematical perspective, a model

can also be built with parameters that are quantitative estimates of real system properties (*e.g.* rates of cell migration or death). These models, referred to as *mechanistic models,* are specifically formulated to provide insight into a biological process, but can be developed only when there is a fundamental knowledge of the interactions between variables of the system (146). For instance, physical systems obey certain fundamental laws (*e.g.* Newton laws) and, so, mechanistic models use these laws to build a description of the system. However, there are no established fundamental laws in Biology (56) or, for that matter, Immunology. Thus, we decided to build an empirical model first because it is mathematically simpler and more objective, *i.e.* it requires making less scientific assumptions. The expectation is that the knowledge acquired in this process may, then, be used to develop a mechanistic model to investigate immunological laws or principles.

The relationship between HDM exposure in a murine experimental setting and humans in the real world is uncertain. Appendix I is a book chapter that includes a comprehensive discussion about this issue (59). In summary, many clinical and epidemiological studies have measured concentrations of mite allergens. However, the site sampled, the sampling techniques used (type of collectors, frequency of sampling, environment), and the methods of measurement and quantification of allergen employed are diverse, leading to variable results. In addition, the relationship between allergen sampled in a given household surface and allergen inhaled is not clear. Despite limitations comparing actual amounts of allergen, it is likely that responses and behaviors can be translated from mouse to human.

Epithelial-associated cytokines in HDM sensitization

The airway epithelium is the site where aeroallergens first interact with the host, and ECs are now recognized as playing important roles in the maintenance of pulmonary homeostasis. In allergic disease, there is increasing evidence that both direct and indirect interactions between ECs and DCs in the airway mucosa regulate innate and adaptive immunity, contributing to Th2 sensitization and progression to chronic airway disease (93,128). Among the soluble mediators secreted by the epithelium, the cytokines TSLP, IL-25 and IL-33 have been the focus of many recent

investigations as their dysregulated production is associated with a number of inflammatory disease states including asthma, inflammatory bowel disease and atopic dermatitis (104). In Chapter 3, we examined the relevance of this triad of cytokines in allergic sensitization to HDM.

At the time of these studies, the prevailing dogma was that TSLP plays a critical role in the generation of allergic disease (110,147,148). In fact, TSLP had been heralded as "the master switch of Th2 responses" (149,150). However, most previous studies had been conducted in conventional models involving intraperitoneal (i.p.) sensitization to a surrogate allergen, such as OVA, adsorbed to aluminum-based adjuvants, conditions inherently limited to elucidate the role of cytokines in Th2 sensitization to mucosally delivered allergens. In contrast, we used a HDM exposure protocol that involves solely mucosal exposure in the absence of exogenous adjuvants. This protocol recapitulates early features of the allergic phenotype which expression is dependent on the canonical Th2-inducing molecule IL-4 (35). Our data showed that, despite increased levels of TSLP in the airways of HDM-exposed mice, TSLP is dispensable for allergic sensitization, Th2 airway inflammation and mucus production. It has been proposed that the ability of TSLP to elicit Th2 immunity is based on its ability to condition DCs by limiting IL-12 production and upregulating MHC II as well as the co-stimulatory molecule OX40L. In a series of *in vitro* studies with bone marrow-derived DCs, we showed that HDM is able to inhibit IL-12 production and upregulate OX40L, thus rendering TSLP redundant. To our knowledge, TSLP-independent Th2 responses have only been reported in certain helminth infection systems (151).

Similar results were obtained regarding IL-25, again at variance with previous reports using conventional OVA models (121,152). In contrast, absence of IL-33 signaling resulted in significant decreases in IgE, airway eosinophilic inflammation, mucus production and Th2 cell activation to HDM exposure. In these studies, we used ST2 KO mice, ST2 being the receptor for IL-33. Upon publication of the manuscript in Chapter 3, we had the opportunity to access IL-33 KO mice and confirmed, in these mice, the responses reported in ST2 KO mice (unpublished data).

We also reported that neutralization of OX40L greatly prevents allergic sensitization and Th2 responses, suggesting that OX40L is a central co-stimulatory signal in HDM-induced Th2 immunity, consistent with a recent publication using OX40 KO mice (42). Besnard *et al.* reported that IL-33 directly stimulates myeloid DCs (mDCs) *in vitro* to express high levels of OX40L (153). We observed that, while TSLP, IL-25 and IL-33 could all similarly induce OX40L on DCs *in vitro*, only IL-33 mediated this effect *in vivo.* Finally, our data showed that IL-33 markedly expands a population of type II innate lymphoid cells (ILCs) in an IL-33-dependent manner during allergic sensitization to HDM. ILCs have been shown to be responsive to IL-25 or IL-33 and produce Th2 cytokines, primarily IL-5 and IL-13 (154). ILCs are known to expand during secondary responses to OVA (155); however, their role in this setting or, particularly, in the initiation of HDM-mediated responses remains to be elucidated. Based on our findings, we propose a model whereby HDM-induced IL-33 upregulates OX40L on DCs and expands ILCs in the lung leading to the induction of robust Th2 immunity.

The relevance of the IL-33-OX40L axis to allergic Th2 immunity is not exclusive to HDM-induced responses. Indeed, we conducted similar studies in a model of peanut (PN)-induced allergy and anaphylaxis. In this model, sensitization is accomplished by the intragastric delivery of PN along with cholera toxin, followed by an i.p. challenge with PN to induce systemic anaphylaxis. These conditions elicit robust immune and physiological responses that are STAT6 and IL-4 dependent, thus implying that the development of PN allergy and anaphylaxis also relies on the induction of canonical Th2 immunity. We showed that PN allergy is, in this model, also TSLP and IL-25 independent. In contrast, absence of IL-33 or OX40L signaling significantly attenuates PN-specific humoral and cellular immunity as well as anaphylaxis. Thus, IL-33 and OX40L appear as critical mediators of not only allergic respiratory responses to HDM but also PN allergy and anaphylaxis elicited through the gastrointestinal route.

The dose utilized throughout the studies in Chapter 3, *i.e.* 25 μg HDM, elicits a maximal response in terms of lung inflammation and markers of systemic Th2 immunity, as shown in Chapter 2. Recent evidence suggests that different HDM

exposure conditions may involve different initiating molecular pathways. For example, TSLP was recently shown to be important for Th2 cytokine production and eosinophilic inflammation only when very high doses of allergen, *i.e.* 100 μg HDM, were administered (156). In this case, HDM was given i.n. once to sensitize and, a week later, mice were challenged for 5 consecutive days. While the exact reason of such dose-dependent effect is unclear, the authors of this work suggest that exposure to high doses of HDM is associated with a mixed Th1 and Th2 phenotype, and speculate that absence of TSLP signaling may further shift the immune response to Th1. In addition, Gregory *et al.* recently showed that neutralization of IL-25 decreases HDM-specific IgE, pulmonary inflammation and Th2 cytokine production in mice receiving 15 μg HDM three times a week for 3 weeks (157). The reasons for such divergences in molecular requirements are unclear but possibly reflect the diversity of pathways available to achieve Th2 immunity, an issue discussed below.

Our studies focused on investigating the role of TSLP, IL-25 and IL-33 on the ontogeny of allergic asthma, particularly allergic sensitization and early inflammatory events. Consequently, our data does not address the potential role of these cytokines in other aspects of the allergic phenotype such as chronic lung remodeling and dysfunction. The study of these outcomes requires longer exposure protocols than the ones we used. Shortly after our manuscript was published, TSLP and IL-25 were shown to be critical in airway remodeling, including collagen deposition and smooth muscle hyperplasia, as well as AHR to HDM (157,158). With respect to IL-33, overexpression in transgenic mice or administration of recombinant IL-33 have been shown to be sufficient for the development of an asthma-like chronic disease with AHR and lung remodeling in mice (109,111), but its relevance on these clinical features upon HDM exposure remains unknown.

Heterogeneity and complexity of allergic asthma

It has become increasingly clear that asthma is a heterogeneous disease. Back in 1947, Rackeman classified asthma into extrinsic (now referred to as allergic) and intrinsic, based on aetiology, *i.e.* the detection or not of a triggering allergen (159), and, later on, antigen-specific IgE. Additional asthma phenotypes based on causation

have been subsequently identified, including aspirin-sensitive, occupational, smoking-associated, viral-exacerbated, obesity-associated or exercise-induced asthma. Asthma management guidelines categorize asthma according to severity of clinical symptoms, *i.e.* intermittent, mild persistent, moderate persistent and severe persistent asthma. Based on the response to therapy, asthma is classified as controlled, partly controlled or uncontrolled, which is a rather inaccurate system of classification as access to healthcare along with patient and physician behaviors contribute to medication use and the severity of the disease. More recently, asthma has been classified according to the predominant type of inflammation (eosinophilic, neutrophilic, mixed or pauci-granulocytic asthma) and has been proposed that these distinctions are helpful to guide the management of the disease. For instance, eosinophilic, but not non-eosinophilic, asthma is associated with a favorable response to corticosteroid therapy. However, all these classification strategies are focused on a single clinical aspect of the disease and, therefore, represent an oversimplification of the complex nature of asthma (160).

Clinical heterogeneity is likely related, at least in part, to molecular diversity and, thus, immunobiological complexity. Regarding genetics, over a hundred polymorphisms that could influence immune responses, pulmonary development or responses to environmental factors have been associated with at least some phenotypic aspects of asthma, such as greater disease severity or IgE expression. The fact that a number of environmental factors, such as exposure to air pollutants or microbes, hormonal factors and obesity, can modulate disease initiation and progression (20) intimates complex gene-environment interactions. Experimental research, particularly in murine models using common aeroallergens, has clearly illustrated the diversity of asthma pathogenesis. For example, as discussed earlier, the requirement of TSLP to induce allergic sensitization is different in OVA-based models *versus* models using HDM and, within these, it varies depending on the degree of exposure. Likewise, the relevance of TLR4 signaling for the induction of HDM-mediated responses including sensitization is highly controversial (our unpublished results and (161-163)). Intuitively, the divergence of results reported can likely be attributed to differences in protocols, including distinct exposure strategies (acute,

chronic, intermittent) as well as types, compositions, amounts and routes of administration of allergen extracts. Perhaps the most informative message from this body of research is that there is, in fact, a diversity of pathways with distinct molecular signatures leading to Th2 immunity. The challenge remains in matching these molecular signatures to discrete clinical phenotypes. It has been suggested that, in this context, the concept of phenotype, which describes observable characteristics with no direct relationship to a disease process, could be replaced by that of endotype, wherein a specific biological mechanism explains the properties of a phenotype (164,165).

The emerging paradigm from molecular, immunologic and clinical studies is that asthma is, rather than a single disease, comprised of a number of disease variants or phenotypes. A precise delineation of these phenotypes will likely have a significant impact on the use of specific therapies. Studies on the efficacy of anti-IL-5 antibodies to control asthma begin to illustrate this notion. Whereas this treatment was ineffective in a general population of asthmatics, it showed significant clinical efficacy in severe asthmatics with prominent sputum eosinophilia (30,166,167). Similarly, one recent analysis of pooled data from trials using anti-IgE antibodies suggests that patients with severe asthma characterized by persistent airflow obstruction, a need for high-dose inhaled corticosteroids and frequent asthma exacerbations are more likely to benefit (168). To date, our understanding of asthma phenotypes remains a work-in-progress. A number of studies have begun to approach phenotyping in a manner that is less biased and more statistically based, including multivariate techniques such as cluster analyses and latent class analyses (169-171). These important contributions regarding clinically-oriented clusters would benefit from incorporating key immunopathological variables, through immune-phenotyping, such as those proposed by Woodruff *et al.* (172), who identified Th2-high and Th2-low asthma, as well as by several experts in the PRACTALL consensus report (165). Arguably, a comprehensive definition of phenotypes based on molecular, immunological and clinical features would allow for the identification of subsets of patients who would benefit from specific therapies and/or management strategies.

Immunological thresholds of responsiveness to HDM exposure

A typical feature of complex, non-linear systems is their sensitivity to initial conditions (173). Therefore, a better understanding of the impact of initial system perturbations might help uncover conditions under which allergic sensitization and asthma may develop. In this context, over 10,000 L of air flow daily through the lungs; the inhaled air contains a plethora of allergens, pollutants, chemicals, microbes and fumes in varying proportions. In Chapter 2 of this thesis, we examined the impact of the dose of HDM in the generation of allergic sensitization and airway inflammation. In Chapter 4, we investigated the impact of concurrent environmental exposures, capable of altering the initial immunological status of the lung, on responsiveness to HDM.

The modeling approach that we used in Chapter 2 allowed us to identify thresholds of responsiveness, understood as the minimum dose of HDM that elicits a measurable response. While thresholds could apply to both the initiation and progression (or exacerbation) of symptoms in allergic asthma, we centered our work in the former. Threshold doses were identified at 0.5 μg of HDM for allergic sensitization (allergen-specific Ig) and 2 μg for airway inflammation. The hypothesis addressed in Chapter 4 was that an initial perturbation, particularly allergen-independent activation of the immune system, would lower the threshold of HDM responsiveness and, thus, increase the sensitivity of the host to develop allergic responses. Experimentally, we chose GM-CSF as the immunological perturbation for several reasons. GM-CSF is a cytokine produced by ECs in response to a variety of inducers, including allergens, bacteria, viruses, fungi, pollutants and irritants (161,174-178). In addition, GM-CSF is capable of stimulating the maturation, proliferation and activation of APCs, notably DCs (53). We used an adenoviral vector system to transiently express GM-CSF in the airway/lung compartment. Our data showed that local and transient expression of GM-CSF facilitates allergic immune-inflammatory responses in mice exposed to sub-threshold amounts of HDM (0.2 μg). This was associated with a marked increase in the number of activated mDCs and enhanced Th2 cell activation and Th2 cytokine production. In addition, we showed

that these responses have long-term consequences, as only mice sensitized with GM-CSF+0.2 μg HDM responded, one month later, to a short re-exposure protocol with drastically low amounts of HDM.

In light of the findings presented in Chapter 3, where we showed that IL-33 plays a central role in HDM-induced responses, we explored the role of this cytokine in a GM-CSF-driven system. Our data showed that GM-CSF overexpression significantly increases IL-33 protein levels in lung homogenates as well as the number of IL-33$^+$ cells in the lung. Interestingly, most of these IL-33$^+$ cells were alveolar epithelial type II (ATII) cells. Other lung cells, including bronchial ECs, endothelial cells and alveolar macrophages, have been shown to express IL-33 in other experimental systems and in humans (107,113,179-182), but they did not immunostained for IL-33 upon either HDM or recombinant GM-CSF exposure. Of note, IL-33 protein accumulated in the nucleus, and we found no evidence for cytoplasmic or extracellular localization. In fact, IL-33 is usually found in the nucleus, where it is thought to act as a nuclear factor and, therefore, regulate gene expression (179). Further research is needed to elucidate how IL-33 is being processed and released in health and disease. Lastly, we demonstrated that exposure to GM-CSF+HDM in IL-33 KO mice results in a considerable decrease in the proportion of OX40L$^+$ mDCs in the lung, and a substantial decrease in airway eosinophilia. Willart *et al.* recently proposed that HDM stimulates ECs to release IL-1α, which, then, acts in an autocrine manner to induce the production of GM-CSF and IL-33 for DC recruitment and activation (156). This group showed that both cytokines are required for HDM sensitization, but did not address the precise sequence of events. In Chapter 4, we evaluated the potential involvement of IL-1α in our model. Stimulation of human ECs with GM-CSF *in vitro* increased IL-33 in an IL-1α independent manner. Likewise, administration of GM-CSF to mice enhanced IL-33 but not IL-1α levels in the lung. Therefore, these data suggest that GM-CSF is upstream of IL-33, and GM-CSF-mediated IL-33 production by ATII cells is independent of IL-1α. Arguably, this is a novel pathway of Th2 immunity in the context of exposure to sub-threshold amounts of HDM. From a larger perspective, these findings highlight the importance of the activation status of the innate immune system of the lung at the time of incipient

allergen exposure, and suggest that a wide array of environmental exposures may increase the susceptibility, *i.e.* lower the threshold of allergen responsiveness, to develop allergic asthma through a GM-CSF/IL-33 pathway.

Knowledge of thresholds in the allergic population may be important to assess public health risk, propose lifestyle changes and building regulations and design preventative strategies. Yet, allergen threshold levels are not well established in the literature. The importance of the role of HDM in allergic asthma prompted the WHO to establish certain guidelines. While different studies provided somewhat different estimates, it was ultimately put forth that Der p 1 levels equal to, or greater than, 2 μg/g of dust (primarily from measurements in mattresses and bedding) are a significant risk for allergic sensitization to HDM; whereas exposure levels above 10 μg Der p 1/g dust are associated with the development of asthma (137,140,183,184). The potential benefit of these recommendations has been questioned by reports that failed to document significant differences in mite allergen levels in homes of children with and without allergic asthma (185-187). Specifically, in the study by Carter *et al.,* dust sampling was not performed only once or twice, as in previous studies, but at regular intervals over a period of 2 years (185), thus incorporating seasonal variations, reducing potential bias, and yielding quite accurate measures of levels of exposure to HDM allergen. While these thresholds may be useful as epidemiological references, it still remains to be clarified how these levels relate to actual amounts of HDM in the air and, eventually, inhaled, and these are surely influenced by many additional variables. An independent but related issue is whether measures to reduce exposure to domestic allergens actually decrease exposure below defined thresholds, and whether such decreases have an impact on clinical outcomes and/or disease incidence. These issues remain controversial (20,22,23,188,189) due, in part, to the need for improved methods to monitor and effectively reduce allergen exposure.

Concluding remarks

The global pattern of disease burden is shifting from infectious pathologies to chronic, non-communicable disorders (2) and, yet, strategies to prevent or treat many of these conditions are limited. This is the case of allergic asthma. We think that this

thesis work contributes to the understanding of the immune dysregulation that takes place during the development of allergic asthma in response to HDM exposure. Our data emphasizes the importance of GM-CSF, IL-33 and OX40L as central mediators of allergic sensitization and asthmatic inflammation, and opens new venues of investigation into their range of activities and potential as therapeutic targets. Moreover, we have provided new tools for the investigation of allergic asthma pathogenesis. In particular, we have incorporated mathematical and computational techniques into immunobiological research, resulting in the identification of system properties and the ability to simulate a wide range of scenarios. In this regard, Aderem and Hood have argued that systems biology, with computer science and applied mathematics as critical tools, will dominate the 21st century as the approach to tackle biological complexity (190). Finally, we have developed a murine model of allergic asthma that mimics instances of concurrent exposure to sub-threshold amounts of HDM with other stimuli capable of activating the immune system. Collectively, these studies support the notion that the development of allergic asthma is highly dependent on allergen exposure and the immunological status of the lung, or host susceptibility, at the time of allergen exposure.

While experimental animal studies have had a major contribution to the understanding of allergic asthma, therapeutic translation has met with considerably limited success. Indeed, quite a number of successful therapeutic interventions in mice have failed in the clinic. Many factors have likely contributed to this discordance. However, the increasingly apparent message from both animal and human studies is that asthma is a heterogeneous disease. Arguably, matching specific animal models with particular human phenotypes will improve the translational potential of pre-clinical research. This effort may result in the identification of individuals, or subsets of patients, who would benefit from specifically targeted therapies.

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(for Chapters 1 & 5)

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Appendix I.

Modeling Responses to Respiratory House Dust Mite Exposure

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Modeling Responses to Respiratory House Dust Mite Exposure

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Abstract

House dust mite (HDM) is the most pervasive indoor aeroallergen source worldwide. Allergens derived from HDM are associated with sensitization and allergic asthma. Allergic asthma is an immunologically driven disease characterized by a Th2-polarized immune response, eosinophilic inflammation, airway hyperreactivity, and remodeling. Animal models of asthma utilizing ovalbumin (OVA) exposure have afforded us considerable insight with respect to the mediators and cell types involved in allergic airway inflammation. However, OVA preparations and HDM are two vastly different materials. This chapter is specifically concerned with modeling responses to HDM exposure in mice. These studies have furnished new information and unlocked new lines of inquiry regarding biological responses to common aeroallergens. The complexity of HDM as an allergen source, with its plethora of protein and nonprotein immunogenic components, may influence the mechanisms underlying sensitization, inflammation and remodeling. Here, we will discuss this issue, along with giving critical thought to the use of experimental models.

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Introduction

Allergic asthma is now understood as an immunologically driven disease that occurs in predisposed individuals as a consequence of aeroallergen exposure. The hallmarks of the asthmatic phenotype principally include the development of a Th2-polarized immune response, eosinophilic inflammation and airway hyperreactivity (AHR); persistent or recurrent inflammation appears to be associated with changes in the structure of the airway referred to as remodeling. This chapter is specifically concerned with modeling responses to exposure

to house dust mite (HDM), the most pervasive aeroallergen worldwide. Throughout the chapter, our hope is to place our discussion in the broader context of modeling of asthma, beginning with a foreword about scientific modeling.

Modeling is an essential part of the scientific endeavor. In medicine, illness is manifested by a collection of signs and symptoms. An observer collects them, appraises them and decides to which recognized pattern they best fit. This pattern recognition exercise, essential in deriving a diagnosis, contributes only modestly to the understanding of the origin of a disease, its evolution and its nature. Origin, evolution and nature are facets of a complex process, especially in the case of chronic diseases such as asthma. Advancing our understanding of these facets will come not only from a thorough study of the literature and much careful consideration but, much more likely, through experimentation. Indeed, it has been largely through experimentation in in vitro systems as well as in in vivo animal and human models that we have learned the most about asthma.

Biomedical modeling generally differs from modeling in other scientific domains. In disciplines such as mathematics, physics and economics, a model is the elaboration of a set of rules governing interactions between different variables. Thus, by definition, there is not a predetermined outcome. For example, the equation that defines Newton's Second Law of Motion, $F = ma$ where *'F'* is the force applied, '*m*' the mass of the object and *'a'* is acceleration, is such a model. These kinds of models are particularly suited to explain and predict the outcome of interactions between a number of variables. In sharp contrast, the outcomes of biomedical models are predetermined; in other words, their goal is to recapitulate a given phenotype. The implication of this is that *one can only model what one knows*. This is what determines the logic of biomedical modeling. The undisputed, but somewhat neglected, corollary of this is that modeling of a disease should be a dynamic and ever-evolving endeavor. An underlying theme of this chapter is that modeling promotes questioning which, in turn, echoes the stage of knowledge at a given point and time.

The notion that one can only model what one knows underlies the development of models of asthma over the last 20–30 years. Inflammation, or features of inflammation, had been noted for a long time in asthmatic individuals. However, the appreciation that eosinophilic airway inflammation resulting from a particular type of immunological reaction is a defining feature of the asthmatic phenotype is more contemporary. This understanding, in conjunction with the explosion of molecular immunology, steered the modeling strategies that researchers chose to explore in recapitulating the asthmatic phenotype, particularly in mice. The benefits of this work are apparent as it is now established that allergic asthma is the product of a Th2-polarized response with all of the attendant implications, including the discovery of the functions of archetypical

Th2 cytokines, notably IL-4, IL-5, IL-9 and IL-13, on cardinal features of the asthmatic phenotype.

The vast majority of research in models of asthma to date has utilized chicken egg ovalbumin (OVA), an innocuous protein, as a surrogate allergen. It appears that early research in anaphylaxis led investigators to make this choice, as there is historical evidence that experimental research in anaphylaxis, notably to food antigens, considerably preceded experimental research in asthma. Indeed, studies of OVA-induced anaphylaxis in animal models date back nearly 100 years [1, 2]. Research into these two processes evolved rather separately for a long period of time. It seems plausible that the realization of the conceptual and mechanistic connections between anaphylaxis and asthma beginning with Meltzer in 1910 [3], along with the easy availability and very low cost of OVA, set the stage for researchers wishing to model asthma to settle on OVA as the 'pseudo-allergen' of choice.

Mouse Models of Allergic Airway Disease

OVA-Based Models of Asthma

There have been many studies, particularly over the last 20 years, in experimental models of asthma. The objective of these initiatives has been to recapitulate the cardinal features of the asthmatic phenotype, notably airway inflammation but also AHR and, more recently, airway remodeling. As indicated earlier, most of the research in this field has been performed in models using OVA, a choice that has fundamentally influenced the strategies employed to generate allergic sensitization. Indeed, the fact that exposure to OVA, either intranasally or by aerosol, to naïve mice elicits inhalation tolerance rather than allergic sensitization or airway inflammation [4–7] has forced researchers to develop ways of circumventing inhalation tolerance. With some minor variations, the most commonly used strategy involves the adsorption of OVA to a chemical adjuvant (usually aluminum hydroxide or alum) that is injected into the peritoneal cavity to generate allergic sensitization; we will refer to these models as 'conventional' models of allergic sensitization from here on. An asthma-like phenotype is, then, elicited by a subsequent airway challenge with OVA. The principal utility of these models resides in the fact that they can generate a consistent and robust inflammatory response and, consequently, allow for the study of the characteristics of such a response. Their greatest limitation is that they are inherently designed to chemically bypass the natural requirements for allergic sensitization, precluding the study of that phenomenon. In addition, neglecting the route by which humans are initially exposed to

aeroallergens – the respiratory mucosa – may have a number of significant immunological consequences.

That allergic asthma is a result of the interaction between inhaled aeroallergens and the immune system brings to the fore the merit of experimental models capable of generating the asthmatic phenotype by exposure through the respiratory route. It seems intuitive that mucosal exposure to OVA, whether via the respiratory or gastrointestinal mucosa, would result in tolerance for there is neither survival advantage nor immunological parsimony in generating a productive immune-inflammatory response against an innocuous protein. A model system was described in which mice were exposed to aerosolized OVA for 10 consecutive days, leading to their sensitization [8]. However, this has not been the finding in most cases. It seems to follow that the lack of inherent immunogenicity of an antigen can be amended by altering the immune status of the host, particularly through the activation of the surveillance apparatus that first interacts with antigens. For example, studies in which granulocyte macrophage colony-stimulating factor (GM-CSF) was overexpressed in the airway milieu enlighten this point because GM-CSF is one of the most powerful natural adjuvants with substantial effects on the maturation and activation of macrophages and dendritic cells. Indeed, it was shown by Stämpfli et al. [9] that GM-CSF enrichment of the airway microenvironment inhibits tolerance induction to OVA and elicits a phenotype with the typical hallmarks of asthmatic inflammation. Mice subjected to 10 OVA aerosolizations in the context of a GM-CSF-enriched microenvironment showed an expansion and activation of antigen-presenting cells (APCs), developed an OVA-specific Th2-polarized immune response associated with airway eosinophilic inflammation and goblet cell hyperplasia, and exhibited AHR. It is likely that molecules other than GM-CSF, such as thymic stromal lymphopoietin, have a direct role in the generation of allergic immune responses [10, 11].

A series of subsequent studies demonstrated that airway overexpression of several cytokines and chemokines such as IL-12, IL-10 and IP-10 were capable of generating distinct immune-inflammatory phenotypes to the same antigen, OVA [12–14]. Together, these studies illustrate that the *context* in which the immune system first sees antigen is of preeminent relevance to the generation of future immune responses and established experimentally, as stated by Lee and Lee [15], the 'macroimportance of the microenvironment'. Yet this line of experimentation was limited, as it did not account for the fact that common allergens are fundamentally different from OVA, and that humans are rarely ever exposed solely to allergens, or to purified allergenic proteins but rather to complex materials. In other words, the biochemical and presumably immunogenic differences between OVA and the allergenic matter humans inhale, such as HDM, may impact not only the outcome of allergen exposure but also the mechanisms by which these outcomes are brought about.

HDM as Allergen

The discovery of *Dermatophagoides pteronyssinus* as the source of the long-sought 'house dust allergen' in 1967 [16] sparked an exponential growth in investigations into the roles of HDM in allergy [17]. Dust mites are ubiquitous throughout humid areas of the world [18]. Of the phylum Arthropoda and class Arachnida, mites are more closely related to spiders, scorpions and horseshoe crabs than they are to insects, and are therefore difficult to control using routine pest control methods. The small size of many mite species (e.g. 20–320 μm for *D. pteronyssinus*, depending on developmental stage) further complicates control of mite populations, all but ensuring exposure. Thirteen different species of mites can be found in house dust, but the three most common species worldwide, and the major sources of mite allergen, are *D. pteronyssinus*, *D. farinae*, and *Euroglyphus maynei*. In tropical climates, *Blomia tropicalis* is also highly prevalent [19]. The discovery of the allergenic role of mites and an understanding of their biology has allowed researchers to address many questions concerning the global distribution of dust allergies, the seasonality of the disease, and the risk associated with damp houses. Many of these issues are still being investigated today.

It has been argued, based on the global prevalence of mite allergens, and the high proportion of total IgE directed towards them, that there is more specific IgE to mite allergens than any other single allergen source in the world [17]. Sensitization to allergens derived from HDM is also strongly associated with allergic asthma, further emphasizing the clinical importance of this allergen source. Much of the knowledge we have in support of which HDM proteins constitute 'allergens' comes from the binding of patient IgE in Western blotting studies. However, these studies are difficult to interpret due to the variation between reports, the potential for cross-reactivity of antibodies, and the lability of antigens [reviewed in 20]. Therefore, identifying which HDM components are the most universally relevant to human disease, and developing appropriate models in which to test hypotheses surrounding HDM allergy, add additional layers of complexity.

Domestic mites are complex organisms that produce thousands of different proteins and other macromolecules. The identification and isolation of mites from dust and their subsequent culture laid the foundation for the development of the extracts currently used in research. HDM extracts are made from an aqueous solution of a variable mixture of whole mites, nymphs, fecal pellets, eggs and spent culture media, all of which are relevant sources of allergenic proteins [21, 22]. Based on a study of these extracts and the high concentrations of these allergens in dust, it is believed that the group 1 and group 2 HDM allergens are 'the major' allergens. However, it is unknown how well these mite extracts actually compare to the allergens present in the environment. It is

possible that some mite proteins present in house dust are poorly represented in extracts due to, for example, their hydrophobicity, stability and/or ability to withstand the extraction process. In addition, extracts differ based on production methods [23], and are difficult to standardize based on the number of proteins they contain [20].

The advent of molecular biology has allowed for the identification, cloning, and synthesis of the proteins identified as HDM allergens. These developments provided the opportunity to investigate the effects of isolated native proteins or recombinant peptides in allergic asthma to HDM. While this approach permits investigation into the effects of a single agent, there are also some shortcomings. First, some recombinant proteins do not retain potentially important native biological activities after being synthesized without a chemical treatment that may affect the protein in other ways. Also, that exogenous adjuvants are required to elicit allergic sensitization to these individual proteins precludes investigation into the immunological mechanisms that underlie this process. In addition, humans are not exposed to a single protein, but to a complex material made up of a multitude of molecules that have the potential to interact. Thus, experiments utilizing a single HDM protein may not capture the complexity of this 'material'. On the other hand, HDM and its extracts are complex; therefore, clarifying the functions of their individual active components and how they contribute to immune responses is a challenging endeavor. This dilemma will likely be mitigated by the convergence of progress arising from the mindful exploration of each experimental paradigm.

Acute HDM Exposure Models

Despite the evidently important contribution of HDM proteins to the myriad of allergens that humans are exposed to, relatively little research has been conducted in animal models to discern the nature of the effects of HDM in vivo. To date, the vast majority of this work has been done using the previously mentioned 'conventional' [intraperitoneal (i.p.) + alum] models of allergic sensitization. Shortly following the discovery of mites as the allergenic source of house dust, and the establishment of culture systems from which to extract mite proteins, research logically focused on the ability to elicit IgE responses in mice and guinea pigs [24–27]. Initially it was discovered that it is possible to sensitize guinea pigs to HDM extracts via the respiratory mucosa [26]. At this time however, sensitization in the absence of adjuvant appeared to be arduous, taking as long as 17 weeks to achieve [24]. In mice, multiple injections of HDM seemed to be required, even in the presence of alum, to generate an IgE response [27, 28]. These early findings appear to have set the stage for the primary use of conventional models of allergic sensitization as the standard in the field. This is true for the majority of studies that have followed, modeling both

allergic asthma and atopic dermatitis, unless modified animals such as T cell receptor transgenics [29] were employed.

Research investigating the immunological mechanisms involved in allergy to HDM began in earnest in the late 1980s to early 1990s. Experiments addressing the allergenic components of HDM extracts have demonstrated that while the higher molecular weight fraction of these extracts (containing Der p 1) does appear to retain the majority of the allergenic potential [30], Der p 1 alone induces oral and inhalation tolerance [31–33]. It is plausible that this finding perpetuated the notion that conventional models were needed to examine the induction of responses to HDM, and the continued use of i.p. models sparked two lines of questioning. One line is concerned with the development models of mucosal sensitization that are more clinically relevant [29, 34, 35], and the other asks immunological questions using conventional i.p. models and the inflammatory responses they elicit. Studies in this latter category have been useful in addressing questions such as the role of eosinophils in AHR elicited by HDM [36], the types of inflammatory responses elicited by different species of mites [37], the role of the proteolytic activity of Der p 1 in inflammatory responses in the lung [38, 39], the importance of the spatial distribution of immune cells in the lungs of primates [40], as well as the feasibility of cytokine gene therapy-based strategies or parasitic infections to treat HDM allergy [41, 42].

As previously mentioned, early attempts to sensitize mice to HDM via the respiratory mucosa were met with limited and inconsistent success. In 1999, Yu et al. [43] demonstrated that sensitization to a crude *D. farinae* extract was possible via the intratracheal route in the absence of exogenous adjuvants. However, the inflammatory responses in the lung and IgE titers obtained were admittedly low, and much more robust responses could be achieved via conventional i.p. sensitization followed by airway challenge. This was followed in 2004 by Cates et al. [44] who published an acute model of allergic sensitization to an HDM extract (*D. pteronyssinus*) in which mice were indeed sensitized via the respiratory mucosa in the absence of exogenous adjuvants. This model was characterized by the current hallmarks of allergic asthma: the expansion and activation of APCs, an eosinophilic inflammation in the lung, deployment of a Th2-polarized immune response, Th2-associated immunopathology and resultant AHR. This acute HDM exposure model also allowed for a preliminary investigation into the roles of a cytokine, GM-CSF, in the early stages of allergic sensitization. In an interesting parallel to the previously reported findings of Stämpfli et al. [9], who demonstrated that enriching the airway environment with GM-CSF allowed for sensitization to OVA, the administration of an anti-GM-CSF antibody in the Cates et al. study markedly decreased responses to HDM. This was believed to be due to the ability of HDM to elicit GM-CSF

production from bronchial epithelial cells [45] and the powerful effects this cytokine has on APCs [reviewed in 46]. Therefore, unlike OVA, HDM appeared to have an intrinsic ability to elicit an immune response, the nature of which can now be studied in more detail. Using a slightly modified model, others later reported similar findings, further expanding their work to examine the impact of regulatory T cells on dendritic cells and allergic responses to HDM [47].

One of the fundamental differences between the models discussed above and allergen exposure in humans is that these animals were exposed to HDM antigens for short, experimentally defined periods of time, whereas human exposure is a ubiquitous life-long event. As such, these kinds of models are inadequate to examine questions surrounding the biological processes that occur as a result of chronic allergen exposure, and if or how these processes play a role in diseases such as allergic asthma.

Modeling the Chronic Aspects of Allergic Asthma

That human exposure to HDM is a chronic event implies that there is a persistent, ongoing interaction between HDM antigens and the immune system. However, the details of the consequences of this interaction in allergic and non-allergic persons are unknown. Currently, it is believed that chronic exposure to allergen results in persistent eosinophilic inflammation, which leads to the structural changes, characteristic of asthma, known as airway remodeling.

Remodeling of the airways was first described quantitatively in 1922 by Huber and Koessler [48], and many subsequent studies have shown this phenomenon to be comprised of increases in mucous production, subepithelial extracellular matrix deposition, and airway contractile elements such as airway smooth muscle cells and myofibroblasts. As the clinical significance of these structural changes to the airway wall has become apparent, efforts have been made to develop model systems to investigate immunological links to this phenomenon.

Many cell types and mediators associated with Th2-polarized immune-inflammatory responses have been implicated, primarily in vitro, in the elaboration of airway structural changes. Cytokines such as IL-13 and IL-4 have been shown to directly effect airway structural cells, leading to increased mucous production [49] and collagen deposition [50], as well as phenotypic changes in fibroblasts [51]. Activated Th2-polarized lymphocytes have also been directly implicated in the increased proliferative capacity of airway smooth muscle cells [52]. Moreover, recent studies into the structural consequences of eosinophil degranulation in the airway have revealed that eosinophil-derived cationic proteins are able to modify gene expression in bronchial epithelial cells, inducing them to synthesize numerous mediators, such as growth factors and matrix metalloproteinases, which ultimately lead to altered extracellular matrix composition and turnover [53]. In addition to the direct impact of inflammatory cells on

airway structure, resident structural cells themselves, including epithelial cells, smooth muscle cells, fibroblasts and myofibroblasts [reviewed in 54], may participate in the initiation of a repair phenotype characterized by increased collagen deposition, which is thought to be driven by TGF-β and other growth factors [55]. Due to the complexity of remodeling, and the number of cell types and mediators involved, the extent and nature of the contributions made by inflammatory and structural cells in airway remodeling and the asthmatic phenotype as a result of chronic allergen exposure in vivo remains to be elucidated.

To address these questions, significant effort has been exerted to recapitulate the chronic aspects of asthma, using animal model systems. One approach to investigating the role of Th2-associated molecules in airway remodeling has been the genetic manipulation of the host. By either upregulating pulmonary expression of key Th2 cytokines such as IL-9 and IL-13 [56, 57] or by knocking out expression of t-bet [58], a transcription factor associated with the generation of Th1 immune responses, chronic airway inflammation, peribronchial fibrosis and abnormal airway function can be established. While these models illuminate the potential role of each of these factors in the structural changes associated with asthma, they ignore the potential contribution of allergen exposure itself on the development and maintenance of abnormal airway structure and function.

Acute protocols employing short-term OVA delivery, even at a high dose, have been used to examine the impact of allergen exposure on airway remodeling. However, these protocols do not lead to most of the structural changes associated with chronic asthma. Therefore, the effects of chronic pulmonary exposure to OVA were explored. Interestingly, exposure under these conditions led to an abrogation of the immune-inflammatory response to OVA. Although initial delivery of OVA results in immune activation, sustained respiratory exposure leads to an attenuation of the immune response to OVA, deviation of the immunoglobulin profile from IgE to IgG1, decreased expression of costimulatory molecules on lung mononuclear cells, and decreased eosinophilia in the peripheral blood and bronchioalveolar lavage, even in i.p. sensitized animals [59].

In order to avoid the development of inhalation tolerance to OVA, various models have been developed in which OVA is delivered intermittently after i.p. sensitization [60–63]. The resultant phenotype of these models includes epithelial lesions (goblet cell hyperplasia, mucous production and epithelial shedding) and alterations to the subepithelium, most notably increased collagen deposition and accumulation of contractile elements (smooth muscle hypertrophy/hyperplasia and myofibroblast differentiation). Many of these models are also associated with increased AHR to methacholine. Interestingly, the extent of eosinophilia subsides progressively with each exposure. In addition, interactions

between many common aeroallergens and the lung microenvironment are a potentially important pathway in the development of altered airway wall structure. This is an aspect that cannot be addressed using OVA as the antigen.

To this effect, models employing chronic exposure to HDM were developed. Recently, in the absence of any adjuvant, chronic exposure to a *D. farinae* extract has been reported to induce airway eosinophilia in infant monkeys [64]. Subsequently, in 2004, Johnson et al. [65] described a mouse model of sustained allergic airway inflammation and remodeling employing chronic, sustained respiratory delivery of a *D. pteronyssinus* extract. This protocol of chronic respiratory exposure to HDM led to sustained airway eosinophilic inflammation, which reached a plateau following 3 weeks of exposure. HDM delivery was associated with immune activation in the lung, characterized by the expansion of APC, activated T cell, and Th2 effector cell populations following 5 weeks of continuous HDM exposure. An allergen-specific memory response to HDM extract was also observed by elevated in vitro production of IL-4, IL-5 and IL-13 by splenocytes isolated from mice chronically exposed to HDM, and humoral immunity to HDM was indicated by elevated serum levels of IgE and HDM-specific IgG_1.

This chronic eosinophilic immune-inflammatory response was also accompanied by profound changes to the structure of the large airways. In addition to the goblet cell hyperplasia and increased mucous production commonly observed in acute allergen exposure protocols, continuous HDM exposure resulted in an increased expansion of peribronchial contractile elements, presumably myofibroblasts, as well as collagen deposition in the airway wall subepithelium. Finally, chronic exposure to HDM had profound functional consequences, with markedly increased AHR to methacholine. Interestingly, the lung structural changes and dysfunction remained following HDM withdrawal, despite an attenuation of the inflammatory response. This suggests that the airway dysfunction seen in this model is contributed to by both airway inflammation and remodeling. It is hoped that a detailed exploration of the evolution of the structural and functional abnormalities in this model will help to improve our understanding of the mechanisms contributing to airway remodeling and dysfunction in the context of chronic exposure to a common aeroallergen.

Considering the Allergenicity of HDM

Clearly, allergic sensitization to a variety of allergens, including OVA and HDM, can be achieved experimentally. These systems do not inform matters such as: (1) what makes allergens like HDM allergenic and (2) under what conditions allergic sensitization naturally occurs. Confronting such questions is the

selective pressure that has driven past changes in modeling and will drive future lines of inquiry.

The Biochemical Nature of HDM Extracts

As previously mentioned, OVA preparations and HDM extracts are, from the perspective of biochemical diversity, two vastly different materials. The OVA preparations that are commonly used in models of allergic disease are typically >98% pure and hence, are essentially free from additional protein and nonprotein components. In stark contrast, HDM extracts are complex materials consisting of hundreds of protein and nonprotein components that range in both size and function. Indeed, it is this fundamental difference between OVA and HDM that is thought to account for the divergent immunological outcomes which arise following inhalation of these materials. This realization has fueled investigations aimed at characterizing HDM-derived allergens and understanding the mechanisms by which these individual components promote allergic disease.

It is estimated that mites produce approximately 3,000 proteins, 5% of which are thought to be allergens [66]. Studies conducted in the mid-late 1980s documented >30 IgE-binding proteins in dust mite extracts using sera from mite-allergic patients [67–69]; however, the actual number of *different* mite allergens represented in those studies may be somewhat less. According to the listing maintained by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies [70], 21 different groups of dust mite allergens have been identified. A biological function has been described for most of these allergens, and collectively, they encompass a broad range of functions including binding proteins, structural proteins, enzyme inhibitors and various enzymes [thoroughly reviewed in 20].

The majority of investigations into the impact of HDM-derived allergens on the pathogenesis of allergic disease have focused on the proteolytic activity of HDM proteins. There are at least 4 HDM allergens that possess proteolytic activity. The group 1 allergens display a mixed cysteine/serine protease activity [71], while groups 3, 6 and 9 are serine proteases [72–74] and a number of mechanisms have been identified by which the proteolytic activity of these allergens is thought to contribute to HDM's allergenicity.

A series of in vitro studies conducted by Wan et al. [75, 76] using solubilized Der p 1 demonstrated that this protease disturbed a monolayer of cultured bronchial epithelial cells, resulting in minor exfoliation and the disruption of tight junctions. Interestingly, this event was associated with increased epithelial cell permeability and transepithelial migration of Der p 1. Similar effects were observed when bronchial epithelial cells were treated with culture medium enriched in serine proteases derived from *D. pteronyssinus* (i.e. rich in Der p 3, 6

and 9) [77]. Together, these results suggest that the proteolytic activity of HDM may facilitate allergen entry into the submucosal environment. Consequently, this may increase HDM's contact with APCs and the likelihood of developing allergic sensitization [76].

In addition to enhanced epithelial permeability, it has become increasingly clear that the proteolytic activity of HDM-derived allergens can have more direct proinflammatory effects. Alveolar macrophages treated with a *D. farinae* extract in vitro respond by producing IL-6, TNF-α and nitric oxide [78]. Similarly, purified Der p 1, 3 and 9 were collectively shown to induce the production of GM-CSF, IL-6, IL-8 and eotaxin from bronchial epithelial cell lines and primary cultures of human bronchial epithelium [45, 79]. Although the precise mechanism of cytokine induction from mite allergen-treated epithelial cells differs depending on the allergen and cytokine, for the most part these responses seem to be mediated by the activation of protease-activated receptor-2 [79, 80]. More recently, a protease-independent mechanism of Der p 1-mediated cytokine release has been reported [81, 82]. Notably, an HDM allergen lacking any known proteolytic activity (Der p 5) was shown to induce cytokine expression from epithelial cells [82]. Taken together, the increased accessibility of allergens resulting from disrupted epithelial integrity, and a proinflammatory microenvironment may strongly promote the development of allergic sensitization. However, it is important to note that the extent to which these events occur in vivo remains unclear.

It has also been suggested that HDM's proteolytic activity may enhance IgE synthesis as Der p 1, in vitro, can cleave the low-affinity IgE Fc receptor (CD23) from the surface of human B cell lines [83, 84]. CD23 is a key regulator of the IgE network as its binding of IgE and IgE-containing immune complexes on the surface of B cells triggers a negative-feedback signal that prevents further IgE synthesis [85, 86]. Moreover, soluble CD23 has been previously reported to enhance IgE synthesis [87–89]. Thus, it has been proposed that Der p 1-mediated cleavage of CD23 may not only impair IgE feedback inhibition pathways but may also augment IgE synthesis directly [83]. Additionally, both HDM extracts and purified Der p 1 can induce the production of IL-4 directly from mast cells and basophils [90], and this may also enhance the IgE response.

The proteolytic activity of dust mite allergens may, furthermore, bias the development of a Th2 response by cleaving the α-subunit of the IL-2 receptor (CD25) on T cells [91]. Schulz et al. [91] have demonstrated that Der p 1-mediated cleavage of CD25 from human peripheral blood lymphocytes resulted in decreased proliferative and cytokine (IFN-γ) responses upon stimulation with anti-CD3 antibody in vitro. Subsequently they demonstrated that Der p 1-conditioned human CD4 and CD8 T cells produce more IL-4, and less IFN-γ,

following stimulation with anti-CD3 in vitro than T cells treated with inactivated Der p 1 [92]. These findings suggested that HDM may limit the development of Th1 response, at the same time promoting the development of a Th2 response and IgE synthesis [91]. Importantly, this notion is supported by in vivo evidence showing that mice immunized with proteolytically active Der p 1 generate higher titers of Der p 1-specific IgE compared to controls immunized with inactivated Der p 1 [93].

The recent discovery of the involvement of chitinases in the pathogenesis of experimentally induced allergic airway inflammation may have uncovered another mechanism by which the functions inherent to HDM proteins may contribute to their allergenicity [94]. Chitinases are enzymes that catalyze the hydrolysis of chitin and are found in arthropods such as dust mites. In addition, a family of chitinase and chitinase-like genes has been identified in both rodents and humans [95–97].

Elias et al. [98] have postulated that some mammalian chitinases may act as potent mediators of the Th2 immune response. In support of this concept, they found increased expression of acidic mammalian chitinase (AMCase) following sensitization and challenge with OVA and, importantly, neutralization of AMCase markedly reduced inflammation and AHR [94]. The expression of mammalian chitinase-like proteins has also been observed in the context of Th2-mediated inflammation during experimental parasitic infection [99]. Moreover, a putative member of the chitinase protein family, ECF-L, may be a potent chemoattractant for eosinophils and T cells [99, 100]. These findings indicate that mammalian chitinases/chitinase-like proteins may play a role in the modulation of the allergic response.

The group 15 and 18 HDM allergens have been identified as chitinases [101, 102]. Based on the findings discussed above, it is reasonable to speculate that the HDM-derived chitinase allergens may function in a manner similar to human chitinase proteins to potentiate Th2 responses. To our knowledge, there is no direct evidence in support of this. However, the group 15 and 18 dust mite allergens show a high frequency of binding to IgE in patient sera, and are the main IgE-binding proteins in allergic dogs [102–104]. This supports the notion that HDM-derived chitinase allergens may influence allergic disease.

Clearly, the functional properties of allergens can profoundly influence their ability to elicit an allergic immune response, although it is likely that in some cases function may be of little significance. For the most part, as it pertains to many of the HDM-derived allergens, this information is simply lacking at this time. For example, the group 10 and 11 allergens have been identified as muscle proteins (tropomyosin and paramyosin, respectively) and have been shown to bind IgE at a high frequency [105, 106]. Similarly, the group 2 allergens bind a high proportion of patient IgE, and have thus been identified as

major allergens. In addition, the group 14 allergens are abundant mite proteins that function as lipid transport or storage molecules [107, 108]. Interestingly, they have demonstrated both potent reactivity in T cell assays and high IgE binding. It remains to be seen if these biochemical functions or immunological activities contribute to HDM's allergenicity.

The complexity of HDM extracts is further enhanced because, in addition to the diverse array of protein allergens, they contain a number of nonprotein components. Of these, lipopolysaccharide (LPS) is the best defined. It is widely acknowledged that LPS is a potent stimulator of the innate immune response and, correspondingly, has powerful adjuvant activity. LPS can elicit the production of a panopy of proinflammatory mediators including cytokines such as TNF-α, GM-CSF, IL-6, IL-8, IL-10 and IL-12 [109, 110], some of which are thought to contribute to the development of allergic responses [110, 111]. That LPS exposure can facilitate allergic sensitization and exacerbate airway inflammation has been demonstrated in experimental systems [112, 113] and moreover, is associated with the severity of allergic airway diseases in humans [110]. Numerous other nonprotein immunostimulators such as lipoteichoic acid and peptidoglycans are abundantly present in the environment, and at least some of these may promote allergic sensitization [114]. However, aside from LPS, very little is known regarding the content of such molecules in HDM. Given their ubiquitous distribution and profound influence on innate immunity, it is likely that at least some of these molecules may also contribute to HDM's inherent allergenicity. Clearly, a comprehensive description of the nonprotein constituents of HDM is required.

It has become apparent that HDM extracts possess, in sharp contrast to OVA, a number of entities capable of distinctly engaging the immune system. What then, could be the immunological relevance of the biochemical differences between OVA and HDM? It seems intuitive that the biochemical and immunogenic profile of HDM extracts will draw on an expanded network of cellular responses and unique molecular signatures. Take for example, the outcome of airway remodeling. While OVA can, under certain conditions (e.g. i.p. sensitization with intermittent exposure) lead to remodeling, achieving the same outcome utilizing an HDM extract and a route of mucosal sensitization does not imply the same underlying biochemical or immunological process; nor can this knowledge be universally applied to the responses elicited by other aeroallergen families. In this regard, preliminary data from our laboratory suggests that in contrast to OVA-based systems [115], TGF-β may *not* be involved in the generation of HDM-induced airway remodeling [116]. So, while some of the experimental outcomes of using OVA or HDM may be quite similar, the mechanistic processes that underlie those outcomes are likely to be fundamentally different.

The Issue of Exposure

A common denominator of the studies cited above is that, in all instances, the concentration of HDM extract administered was experimentally determined by the researchers to achieve their desired outcome (e.g. 25 μg/day/mouse). Therefore, a legitimate question is: how comparable is this concentration of allergen to human exposure? Absolutely the question is intuitive and legitimate; however, the answer is just as elusive. Arguably, the significance of this issue is relative, because *a model of a chronic disease such as allergic asthma is the compression in time and space of a protracted process*. '*In time*' because the objective is to achieve a predetermined outcome in a relatively short period of time; indeed, the alternative of achieving such a goal in 1–2 years would be a self-defeating enterprise. '*In space*' because, clearly, 'real life' is not the same as life in a cage in an animal facility or, for that matter, the environment in a university research laboratory (in the case of human research). These are the inherent limitations of experimental modeling where the trade-off between 'real life' context and the control of variables and productivity must be constantly scrutinized. The relevance of the question of comparative exposure is also dependent on the nature of the overall research question being asked. If the query is: what concentration of, and under what conditions, an HDM extract elicits allergic sensitization and/or airway inflammation, then the information derived from using a single concentration of 25 μg is largely uninformative. Studies comprehensively examining a wide range of doses and lengths of exposure would shed light on these questions. However, if the issue is to consistently establish a robust phenotype with the typical hallmarks of asthma, then, that concentration is suitable. Nevertheless, these comments should not be construed as intellectual trickery to circumvent the genuine relevance of the initial question: how comparable is this experimental dose of allergen to human exposure? The reply is saturated with uncertainty because the terms of reference are ambiguous. Indeed, many clinical and epidemiological studies have measured concentrations of mite allergens. However, the site sampled, the sampling techniques used (type of collectors, frequency of sampling, environment), and the methods of measurement and quantification of allergen employed are diverse. Moreover, the criteria employed to define 'risk' (for sensitization as well as disease) are not uniform. Therefore, to precisely identify the concentrations of HDM associated with allergic sensitization and airway disease in humans is an arduous endeavor.

With respect to allergen sampling, there is still debate as to whether *indirect* personal exposure should be assessed by measuring allergen, usually Der p 1, either in airborne dust, in settled dust, or in allergen reservoirs [117]. *Airborne allergen* has been commonly measured by air samplers or ion-charging devices [117, 118]. Under these conditions, allergen is usually expressed as nanograms

per cubic meter of air. Most investigators have been unable to detect airborne mite allergens in the absence of experimental or household disturbance, likely because these levels are below the detection limit for most assays. Allergens in *settled dust* can be detected and measured by leaving Petri dishes out for 2 weeks or using an adhesive-membrane system [117]. In this case, allergen-settling rate is calculated as nanograms per square meter per day. A third method used to measure allergen concentrations is to sample *allergen reservoirs* by means of vacuum sampling [117]. In this instance, exposure to indoor allergens is usually expressed as the concentration of allergen detected in dust samples (μg/g dust); this is considered to be the most consistent method available [119]. Despite controversy as to how the allergen concentrations measured using these varied sampling methods might correlate with each other [117, 120], measurement of mite allergen concentration in dust reservoirs, as a surrogate for airborne exposure, is the most commonly used method.

The site of allergen sampling is also relevant. Mattresses, carpets/floors and upholstered furniture are the best places indoors to detect mites and mite products. Other places with adequate textile substratum and optimal temperature, humidity, and food resources for mites are shutters, clothing and picture frames [121]. It has been proposed that mattress-dust sampling is the most appropriate marker of indoor allergen exposure because bedding is a major reservoir of mite allergens and probably the most important environment for exposure. A number of studies have documented that the amount of mite allergens found in mattresses is up to 10-fold higher than in other areas of a household [122, 123]. It should be noted that the distribution of allergen is dynamic. In particular, the concentration of airborne allergen is notably influenced by any 'disturbance factor'. For example, it has been shown that the airborne levels of Der p 1 increase about 1,000-fold in disturbed conditions, such as bed making [122] or vacuum cleaning done without a filter.

With these considerations in mind, many clinical and epidemiological studies have examined the amount of mite allergen present in homes. The central message that emerges from these studies is that there is an extraordinary geographical variability in terms of both the number of households where mites can be detected and the concentration of mite proteins detected at each site. A recent study by Zock et al. [124] examined over 3,500 households in 22 European cities representing 10 countries. The percentage of households in which mite allergens were detected ranged from 0 to 98.4% (globally, an average of 48.4%). Detection of mite allergens was performed by vacuuming 1 m^2 of bed for 2 min; under these conditions, the concentration of mite allergens detected (Der p 1 and Der f 1) ranged from 0.01 to 14.71 μg/g of dust. Other European studies have found concentrations of mite allergens ranging from 0.18 μg/g of dust (carpets) and 5.6 μg/g of dust (mattresses) in Germany [125],

to 16 and 28 μg/g of dust in Southern England and Manchester [126, 127], respectively. Leaderer et al. [128] showed that 47% of households in Connecticut and Central Massachusetts had levels of HDM greater than or equal to 2 μg/g of dust, and in 22% of households levels were greater than or equal to 10 μg/g of dust. Higher levels were reported in coastal Australia and New Zealand where prevalence and severity of asthma is the highest [129]; indeed, a study in Wellington reported that at least 2 μg/g of dust were detected in all households, and 36% had levels greater than 100 μg/g of dust [130, 131]. Finally, many studies have shown that there is a plethora of variables influencing the concentration of mite allergens in the household including humidity in particular, temperature, living conditions such as dampness, number of people living in a household and availability of central heating, living habits such as vacuuming mattresses and sleeping with the window open in winter, and many others [119, 124, 132–134]. Some of these variables not only influence the amount of mite allergen that can be detected but also they are likely to alter the actual amount of airborne allergen.

It is, at this point, tempting to furnish an answer to the question of how daily human exposure to mite allergens compares to the daily exposure of mice to these same allergens in experimental systems. The amount of HDM that interacts with the respiratory mucosa of the lower airway of a mouse may be 5- to 10-fold less the amount deposited into its nostrils [135]. Despite this degree of fluctuation, the actual amount of HDM a mouse is experimentally exposed to per day can be reasonably estimated. Clearly, it is far less direct to deduce the amount of allergen interacting with the lower airway respiratory mucosa of a human on a per day basis from the number of micrograms of Der p 1 per gram of collected dust. For example, that several micrograms of Der p 1 can be measured in a sample that has been collected by vacuuming 1 m^2 of a mattress for 2 min says little about how much was made airborne, inspired, and passed the necessary physical barriers to reach the lower respiratory tract. In other words, the logical ambiguity in precisely determining mucosal exposure to HDM in humans precludes the beneficial intent to formulate a rigorous interspecies comparison of exposures.

A different but relevant issue is the relationship between levels of exposure and the risk for sensitization, an altogether different issue to that of the relationship between allergen exposure and allergic disease. While some controversy remains, the consensus is that there is an association between measurable levels of HDM allergen and prevalence and severity of allergic sensitization [136–138]. However, whether this relationship is linear remains to be elucidated. Different studies have provided somewhat different estimates, but it has been put forth that Der p 1 levels as low as 2 μg/g of dust are a significant risk for allergic sensitization [119, 139]. While such measurements are clearly

useful as an epidemiological reference in defined settings, extrapolation of detected amounts of Der p 1 in dust to explaining pathogenesis may extend beyond applicability. That some studies have failed to uncover a relationship between the concentration of mite allergens per gram of dust and the extent of allergic sensitization is not highly surprising, as there may be a number of reasons accounting for this apparent disconnect. For example, humans are not really exposed only to Der p 1, or Der f 1, but to complex materials, which, as previously discussed, contain other immunologically active moieties. Thus, the mite materials humans are exposed to 'in the wild' are more than likely to be rather heterogenous. In addition, immune responsiveness to HDM (or to Der p 1) is surely influenced by the immune status of the host at the time of exposure. For example, concurrent exposure to other immunologically active entities such as molds and viruses, preexisting sensitization to other non-HDM allergens, or exposure to pollution among other things may prime the immune system altering, either way, the threshold of responsiveness to mite allergens. This complex interplay would directly impact on the concentration of HDM (or Der p 1) required to elicit allergic sensitization. In any case, failure to find a correlation between allergen exposure and sensitization does not justify the conclusion that these two variables are not related.

Closing Remarks

Evidently, modeling is pivotal to advancing knowledge in any scientific discipline. In the medical sciences, experimental modeling aims to recapitulate a predetermined outcome. While the generally implied goal of modeling is to 'mimic a disease', we must be mindful that this is a misguided enterprise. Overall, disease is an experience that is virtually impossible to duplicate, especially across species. Indeed, the experience and manifestation of disease have not only many anatomical and physiological constraints but also fundamentally distinct social and psychological aspects, all of which have an impact on the immune system. Perhaps, the most constructive way to conceptualize modeling is that it is an attempt to recapitulate the phenotype that we think, *based on our best understanding at a given point in time*, underlies a disease state.

Indeed, the notion of '*our best understanding*' does not refer only to the amount of information available at any point in time. Of course information plays a central role but equally, if not more important, is the current conceptualization, the established paradigm that 'explains' the disease. This, we surmise, is what fuels future and novel modeling initiatives. In other words, if asthma is understood, as it was historically, as a 'nervous imbalance', or a 'mediator hyper-release process', then modeling will go along one path. If, in contrast,

asthma is understood as an immune-driven disease, then modeling will evolve along a very different path, both practically and conceptually.

A number of experimental models of asthma have been developed to date. The past, present and future usefulness of each one of them rests in the ability of researchers to remain both cognizant of the limitations of each experimental construct, and open to the changes that must occur with time and discovery. The greatest liability of exploiting a model uncritically is that it may end up furnishing knowledge alienated from, and hence of doubtful relevance to, the very disease process it was meant to investigate.

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Appendix II.
Interleukin 3, Interleukin 5, and Granulocyte–Macrophage Colony-Stimulating Factor

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14 Interleukin 3, interleukin 5, and granulocyte–macrophage colony-stimulating factor

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Introduction

Survival of an organism depends on its ability to mount an immune-inflammatory response that is specific and sufficiently measured to avoid immunopathology. Such a response is highly complex as it involves many exquisitely coordinated steps, from the generation of the set of innate signals that will instruct an effective adaptive response to the mobilization of particular subsets of leukocytes from the bone marrow into the blood and, ultimately, into the tissue, where they are activated to fulfill their effector functions. Interleukin 3 (IL-3), IL-5, and granulocyte–macrophage colony-stimulating factor (GM-CSF) are three cytokines produced by myeloid and structural cells that participate in many steps of this complex process. They exhibit pleiotropic effects, including proliferation, differentiation, survival, and activation of several hematopoietic cell lineages and their precursors, and mediate a number of overlapping and distinct actions in the early and late stages of allergic inflammatory responses. This chapter will first outline some of the structural and functional features of IL-3, IL-5, and GM-CSF. Then, it will examine the expression of these cytokines in clinical settings and follow with a discussion of the immunobiologic functions of each of these cytokines both *in vitro* and *in vivo*. This chapter will conclude with an appraisal of the potential of the therapeutic strategies aimed at inhibiting the activity of IL-3, IL-5, and GM-CSF in allergic diseases.

Structure of IL-3, IL-5, and GM-CSF, and their receptors

IL-3, IL-5, and GM-CSF are 133–166 amino acid-long glycoproteins. IL-3 and GM-CSF are biologically active as monomers, while IL-5 requires homodimer formation given its inactivity as a monomer. They belong to a family of hematopoietic cytokines, with a likely common ancestral relationship given their genetic proximity on chromosome 5 in humans and chromosome 11 in mice. Their gene expression is in part regulated by conserved DNA elements, including cytokine-1 and conserved lymphokine elements, as well as the transcription factors NFAT, AP-1, and NF-GMa, among others. Each of these cytokines, however, can also be differentially regulated by distinct transcription factors and enhancers.

The receptors for IL-3, IL-5, and GM-CSF are glycoproteins belonging to the type I cytokine receptor family. They are composed of a heterodimeric complex of two single span transmembrane polypeptides: a unique α subunit (IL-3 receptor α [IL-3Rα] or CD123, IL-5Rα or CD125, and GM-CSFRα or CD116) and a common β chain (βc or CD131) [1,2]. The α and βc chain genes are encoded on different

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chromosomes and, unlike humans, mice have two different receptors for IL-3 owing to the presence of two β chain genes: a β_{IL-3}, which dimerizes only with the mIL-3Rα, and a βc, which functions similarly to the human βc [2,3]. The α chains are cytokine-specific and can bind their cognate ligand with low affinity, forming binary complexes (ligand:Rα). They have conserved motifs in the cytoplasmic domain that seem necessary for signal initiation but do not transduce any biologic activity [4]. It is the recruitment of free β chains that increases the cytokine affinity of intermediate complexes (ligand:Rα:β), which dimerize to form mature hexamer complexes (2ligand:2Rα:2β), and induce signal transduction [4].

A number of *in vitro* studies have suggested hierarchical receptor binding, or cross-competition, of these cytokines in human cells. For example, in eosinophils, GM-CSF is the strongest recruiter of βc followed by IL-3 and then IL-5; in basophils, IL-3 outcompetes GM-CSF and IL-5 for βc binding [5,6]. In addition, different cell types show low- and/or high-affinity receptors based on the α subunit to βc expression ratio. For instance, eosinophils express receptor α chains of GM-CSF, IL-3, and IL-5 in similar numbers to βc, displaying only high-affinity binding; monocytes, which do not bind IL-5, express both low- and high-affinity receptors for GM-CSF and IL-3 since their respective α chains are present in excess over βc [7]. IL-5Rα and GM-CSFRα also exist as secreted soluble isoforms. They may compete with their respective transmembrane Rα for cytokine binding, potentially contributing to downregulation of IL-5 and GM-CSF responses [7,8].

Pleiotropy, redundancy, and specificity of IL-3, IL-5, and GM-CSF

IL-3, IL-5, and GM-CSF are pleiotropic cytokines because they can each elicit multiple functions [9]. Originally classified as hematopoietic cytokines based on a number of *in vitro* and *in vivo* studies demonstrating effects on the differentiation, growth, survival, and activation of progenitor cells, they have been shown also to modulate a variety of activities in mature leukocyte populations. From a molecular perspective, pleiotropy can be explicated by the presence of IL-3R, IL-5R, and GM-CSFR on multiple cell lineages and the ability of these cytokines to activate multiple signaling pathways, namely Jak/STAT, PI3K/PKB, and several MAP kinase (Ras-Raf-ERK, JNK/SAPK, and p38) pathways [10].

The fact that IL-3, IL-5, and GM-CSF share the βc as well as several signal-transduction pathways leads to a degree of functional redundancy. Teleologically, pleiotropy and redundancy might be viewed as protective fail-safe immune mechanisms to ensure survival in the event of failure of a single pathway. While this is a seemingly undesired sequela in the case of allergic disease, we must be mindful that the underlying mechanisms that operate in response to allergens are likely developed, ontogenically, to fight against pathogens, notably parasites [11]. Despite a degree of redundancy, it is clear that IL-3, IL-5, and GM-CSF retain functional specificity. This functional specificity can be accounted for by several mechanisms, such as the existence of several βc cytoplasmic domains and the distribution of nonshared receptor α subunits in different cell types or at different stages of differentiation. Interestingly, specificity in GM-CSF signaling is plausibly regulated by a phosphotyrosine/phosphoserine binary switch phenomenon [12]. Depending on cytokine concentrations, either one of two mutually exclusive positions in the cytoplasmic tail of the GM-CSFRβ, Ser585 or Tyr577, are phosphorylated to independently promote cell survival alone or cell survival and proliferation, respectively [12].

IL-3, IL-5, and GM-CSF in steady-state and emergency responses

Despite a plethora of findings *in vitro*, IL-3, IL-5, and GM-CSF do not play a major role in steady-state hematopoiesis *in vivo*. Typically, this is illustrated by studies in βc-deficient mice (which allows for IL-3 function via the β_{IL-3} subunit) and βc/IL-3 double-knockout mice [13–16]. Mice lacking the entire IL-3/IL-5/GM-CSF signaling pathway develop normally and have entirely normal hematopoiesis with the exception of decreased basal eosinophil numbers. In addition, these mice show normal hematopoietic recovery after treatment with the cytotoxic drug 5-fluorouracil [13].

In contrast to steady-state hematopoiesis, these cytokines play important roles in pulmonary homeostasis. βc/IL-3 double-knockout mice exhibit evidence

of pulmonary peribronchovascular lymphoid infiltrates and pulmonary alveolar proteinosis (PAP)-like disease, which consists of enlarged foamy alveolar macrophages and an overabundance of surfactant proteins and phospholipids [14–16]. Studies in GM-CSF-deficient mice or involving expression of GM-CSF in lung epithelial cells demonstrated that the absence of GM-CSF was the principal cause of this phenotype [17,18]. In accordance with the experimental data, patients with point mutations or exon deletions in the gene encoding βc or GM-CSFRα, or with circulating neutralizing autoantibodies to GM-CSF, have no apparent hematopoietic abnormalities but do exhibit PAP [19,20]. Lastly, Uchida *et al.* [21] provided evidence in humans with PAP and in GM-CSF-deficient mice of neutrophil dysfunction, including reduced bactericidal capability, which could contribute to their increased susceptibility to a wide variety of infections.

Of particular relevance to T helper type 2 (Th2) immunity, βc-deficient mice exhibit a reduced number of eosinophils not only at baseline but also in response to infection with the nematode *Nippostrongylus brasiliensis* [14]. In addition, $\beta c/\beta_{IL-3}$ double-knockout mice exposed to ovalbumin (OVA) fail to mount an eosinophilic response in the lung and have reduced myeloid dendritic cell (DC) numbers, Th2 cell proliferation, cytokine- and OVA-specific immunoglobulin E (IgE) production as well as decreased airway hyperresponsiveness (AHR) and mucus hypersecretion [22]. Collectively, the evidence reveals the nonessential role of IL-3/IL-5/GM-CSF signaling in steady-state hematopoiesis but emphasizes its importance in lung homeostasis and response to some pathogens and allergens.

IL-3, IL-5, and GM-CSF in allergic airway disease

At the genetic level, a single nucleotide polymorphism in the IL-3 gene has been significantly associated with the risk of nonatopic asthma and atopy in nonasthmatics. Also, polymorphisms in the GM-CSF gene have been associated with atopy and atopic asthma [23]. Likewise, there have also been reports of polymorphisms in IL-5 and IL-5Rα linked to allergy. Numerous studies have demonstrated the presence of IL-3, IL-5, and GM-CSF in cells, secretions, and tissues of patients with respiratory allergic diseases. Furthermore, fluctuations in their level of expression in relation to disease severity, natural exacerbations, and experimental allergen challenge have been amply documented. However, these studies establish merely correlative relationships. Investigation of the role of these cytokines in experimental systems of disease, which will be addressed below, aids to advance causality arguments and uncover the specific mechanisms underlying their effects.

Immunobiologic functions of IL-3, IL-5, and GM-CSF

Interleukin 3

IL-3 was originally defined as multilineage colony-stimulating factor (multi-CSF) with potent growth factor activity for both human and murine progenitor cells. Subsequently, IL-3 was shown to be a differentiating and activating factor for a number of cell types, particularly basophils, mast cells, and eosinophils. Several hematopoietic cells, including T cells, NK cells, mast cells, and basophils are capable of secreting IL-3. In particular, both Th1 and Th2 $CD4^+$ T cells can produce substantial amounts of this cytokine upon activation via TCR/CD3. With respect to mast cells and basophils, IL-3 production is linked to IgE/FcεRI-dependent activation or stimulation with calcium ionophores.

Short-term treatment of mice with IL-3 specifically induces an expansion of basophils [24]. Similarly, IL-3 administration to healthy nonhuman primates leads to marked increases in blood leukocyte counts, particularly basophils and eosinophils [25]. Additionally, the long-term culture of murine bone marrow cells with IL-3 leads to the emergence of eosinophils and mast cells [26,27]. In regards to *human* mast cells, the role of IL-3 on differentiation is controversial and sometimes discrepant with murine data; indeed, divergent effects have been reported depending on the tissue. Lastly, recent studies have shown that IL-3 is able to promote the development of peripheral blood monocytes to macrophages and myeloid DCs [26,28].

In addition to its effects on cell differentiation and expansion, IL-3 can regulate a number of biologic activities. For example, IL-3 induces accumulation of histamine in basophils [25]; while it does not signifi-

cantly stimulate its release in basophils from healthy individuals, it does so in basophils from allergic subjects. IL-3 is also capable of enhancing cytokine secretion, including IL-4 and IL-13, from human basophils [29,30]. Interestingly, a recent study has reported a role for IL-3 in the recruitment of basophils into the lymph nodes, but also a dispensable effect in the development of Th2 immunity following helminth infection [31]. Furthermore, studies *in vitro* have demonstrated an effect of IL-3 on eosinophil, plasmacytoid DC, and macrophage survival.

While hematopoiesis is unimpaired in IL-3-deficient mice, some forms of delayed-type hypersensitivity as well as parasite immunity, including worm expulsion, are compromised owing to decreased numbers and activation status of immune-effector cells [32–34]. In an experimental model of allergic asthma, administration of recombinant IL-3 to sensitized rats led to a doubling in the number of mast cells in the airways and increased eosinophilia after OVA challenge, yet lung resistance after challenge was not altered [35]. In mice, IL-3 neutralization after allergen challenge increased leukocyte clearance by apoptosis and reduced eosinophil activation, the number of infiltrating eosinophils to the airways, and AHR [36]. In sharp contrast, Hsiue *et al.* [37] have shown that IL-3 neutralization in guinea pigs sensitized and challenged with a crude mite extract exhibit a similar degree of eosinophilic airway inflammation as similarly treated controls.

Interleukin 5

IL-5 is associated with an array of activities including the promotion of differentiation, proliferation, migration, survival, and activation of several hematopoietic cells, namely B cells, basophils, and eosinophils. While Th2 cells are the main source of this cytokine, several other cell types can also produce IL-5. These include bronchial and nasal epithelial cells in response to NO_2, O_3, and *Staphylococcal* enterotoxin B; mast cells in response to lipopolysaccharide (LPS) and IgE cross-linking; and airway smooth muscle (ASM) cells, basophils, eosinophils, and $CD34^+$ progenitor cells in response to various intrinsic stimuli, such as cytokines and chemokines.

It is well established that IL-5 may drive the differentiation of activated B cells into antibody-secreting plasma cells (ASCs). Correspondingly, complementary DNA microarray analyses showed that IL-5 regulates many Ig-related genes as well as genes involved in B-cell maturation [38]. Additional studies demonstrated that IL-5 also contributes to isotype class switching [38,39]; however, this effect may be limited to the IgG isotype as other isotypes do not appear to be inducible by IL-5 [39,40]. Moreover, many *in vitro*-based studies have demonstrated that IL-5 significantly enhances B-cell antibody secretion, although this effect may too be restricted, in this case to B-1 and not B-2 cells. In agreement, IL-5 overexpressing transgenic mice exhibit markedly elevated levels of serum Igs [41], and loss-of-function studies have demonstrated that, in the absence of IL-5, homeostatic proliferation and survival of mature B-1 cells are significantly reduced [42].

That IL-5 can also induce the differentiation of eosinophil/basophil progenitors and some cell lines into basophils, at least *in vitro*, suggests that IL-5 also acts as a basopoietin [43], although the findings of Saito *et al.* [44] indicate that this effect may not be critical *in vivo*. Arguably, the most significant contribution of IL-5 to allergic airway disease stems from its effects on eosinophils. Initially, IL-5 was shown to induce eosinophil differentiation from human bone marrow, and, accordingly, was named eosinophil differentiation factor. In concordance, IL-5 transgenic mice display a massive increase in the number of circulating eosinophils [41]. In addition, IL-5 is known to be a chemotactic factor for eosinophils and may augment eosinophilic migration toward other chemotactic factors. IL-5 may further contribute to eosinophil migration into the lung by enhancing adhesion to, and diapedesis through, the endothelium. Also, many *in vitro* studies have demonstrated that IL-5 activates eosinophils and induces the production of various compounds including cytokines, growth factors, vasoactive mediators, and toxic products, implying that eosinophils may drive various aspects of the allergic response, including inflammation, remodeling, and bronchoconstriction. Of significance, IL-5 may further influence airways responsiveness in an eosinophil-independent manner by directly affecting ASM cells. Indeed, ASM cells express IL-5Rα substantially [45], and their incubation with IL-5 and acetylcholine results in enhanced contraction compared with acetylcholine alone [45]. IL-5 also acts to promote eosinophil survival by inhibiting the induction of apoptosis [46]. Interestingly, eosinophils were

found to downregulate cell-surface expression of the IL-5Rα subunit following stimulation with IL-5, suggesting that, at some point, eosinophils may become insensitive to the effects of IL-5 [47].

Thus, IL-5 is thought to play an important role in allergic airway disease via its effects on eosinophils. The question of whether eosinophils play a role in asthma is perhaps one of the most debated issues in the field. Certainly, a considerable number of experimental studies have examined the role of eosinophils in all facets of the asthmatic response using animal models. Collectively, the evidence suggests that eosinophils are not fundamentally required for the development of allergic sensitization [48,49]. Regarding inflammation, the findings indicate that eosinophils may augment leukocyte recruitment and mediator release, although the exact extent of their contribution appears to vary depending on the experimental system and ranges from little, if any, to considerable. Nearly two-dozen studies in experimental animal models have investigated the involvement of eosinophils in AHR. The results of these studies are split, with half showing that eosinophils do play a role in this process and half do not [49]. Similarly, the impact of eosinophils on the development of airway remodeling remains somewhat unclear [49]. Several explanations have been proposed in an attempt to resolve these discrepancies, although, ultimately, none of these explanations have proven entirely adequate [48,49]. Noteworthy, the use of IL-5-based interference strategies as a means of clarifying the role of eosinophils in allergic airway disease is complicated by the fact that IL-5 is known to be involved in other processes that may impact disease in an eosinophil-independent manner.

Granulocyte–macrophage colony-stimulating factor

In 1977, Burgess *et al.* [50] were the first to purify GM-CSF, also known as CSF2, from mouse lung-conditioned medium. Originally defined by its capability to generate both granulocyte and macrophage colonies from precursor cells in mouse bone marrow, it had become evident that GM-CSF can mediate many biologic effects, including the promotion of differentiation, proliferation, migration, survival, and activation on several hematopoietic cells such as monocytes, macrophages, granulocytes, and DCs.

With respect to cellular sources of GM-CSF, several studies in patients with asthma have detected, in agreement with *in vitro* findings, significant upregulation of GM-CSF, either mRNA or protein, in bronchial epithelial cells, lymphocytes, and alveolar macrophages. There is extensive evidence demonstrating that the production of GM-CSF can be directly as well as indirectly induced by a wide array of stimuli. For example, regarding microbes, several studies have shown that GM-CSF production can be induced by a number of bacteria, fungi, and viruses, including *Mycobacterium tuberculosis*, LPS, *Pneumocystis carinii*, *Aspergillus fumigatus*, rhinovirus, and respiratory syncytial virus. The relevance of the role of GM-CSF in host defense is demonstrated by evidence of impaired macrophage clearance of group B *Streptococcus* or *Pneumocystis carinii* following infection in GM-CSF-deficient mice and recovery after administration of recombinant GM-CSF. Non-biologic, harmful entities such as urban air pollutants (e.g., ozone, nitrogen dioxide, suspended particulate matter, and its main component, diesel exhaust particles) have been shown to induce expression and production of GM-CSF by airway epithelial cells and alveolar macrophages both *in vitro* and *in vivo* [51]. Common biologic environmental allergens such as the house dust mite (HDM) allergens Der p 1, 3, and 9 can induce GM-CSF production by human bronchial epithelial cell cultures [52]. In addition to microbes, pollutants, and common environmental allergens, a number of *in vitro* studies have shown that GM-CSF production by human hematopoietic progenitor cells, basophils, neutrophils, bronchial epithelial cells, ASM cells, endothelial cells, and fibroblasts can be induced after stimulation with several inflammatory mediators such as TNF-α, IL-1, and IL-33. That GM-CSF can be potentially induced by such a variety of intruders as well as by secondary signals elicited in the tissue intimates that GM-CSF plays a central role in both innate and adaptive immunity.

In animal models of OVA-induced allergic asthma, neutralization of GM-CSF, or the use of GM-CSF knockout mice significantly attenuates key hallmarks, such as inflammation, eosinophilia, mucus production, and AHR [53,54]. On the other hand, GM-CSF overexpression via the delivery of an adenoviral vector expressing the GM-CSF transgene at the time of challenge resulted in a more sustained accumulation of eosinophils, macrophages, neutrophils, and

proliferating lymphocytes in both bronchoalveolar lavage and airway tissue [55]. Also, GM-CSF expression in the lungs can subvert the development of inhalation tolerance to OVA and instead facilitate the development of an asthmatic phenotype [56]. More recent studies using common environmental allergens such as HDM and ragweed extracts, which can mimic many features of allergic asthma when delivered to the respiratory mucosa in the absence of exogenous adjuvants, have strengthened the role of GM-CSF in allergic responses [57,58]. Thus, many studies strongly support the notion that GM-CSF plays a central role in facilitating allergic sensitization, plausibly through its effect on antigen-presenting cells (APCs). In addition, GM-CSF contributes to the enhancement and/or maintenance of airway inflammation through its effects on the proliferation, migration, function, and/or survival of immune-inflammatory cells such as eosinophils and lymphocytes and, hence, on allergic responses.

Intervention studies in humans

Inhibition of IL-3 bioactivity using monoclonal anti-IL-3 or anti-IL-3Rα antibodies, IL-3 antisense oligodeoxynucleotides, or a recombinant IL-3 fusion immunotoxin has been investigated in individuals with leukemia with some promising findings. However, to our knowledge, no clinical trials have been reported studying the impact of IL-3 neutralization in allergic diseases. Similarly, no studies have investigated GM-CSF neutralization in allergic diseases, most likely because such an approach could severely hamper host responses to harmful intruders and compromise pulmonary homeostasis.

In contrast, several clinical trials have evaluated the therapeutic potential of IL-5 inhibition [59–64]. As expected, treatment of asthmatic individuals with the anti-IL-5 antibodies exhibited long-term decreases in blood and sputum eosinophils, thus demonstrating a role of IL-5 in human eosinophilopoiesis [60,62,63]. However, this did not translate into clinical or functional improvement since parameters of asthma expression, such as FEV_1, airway responsiveness to histamine, symptom scores, and allergen-induced late asthmatic response, were not affected by the treatment [60,62,63]. This disconnection between decreases in the number of eosinophils and unaltered clinical and physiologic responses instigated a questioning of the relative importance of eosinophils in asthma pathogenesis [65]. However, alternate explanations have been proposed to clarify that divergence. For example, airway biopsy samples revealed that the decrease in eosinophils in the lung tissue itself was actually modest following anti-IL-5 treatment [60,61]. Additionally, the treatment did not affect levels of eosinophil major basic protein, intimating a further disconnection between eosinophil numbers and degranulation.

While issues of sample size arose with the earlier clinical trials, a recent study involving 300 asthmatic individuals with a spectrum of mild to severe disease generally agreed that anti-IL-5 therapy was unsuccessful [59]. However, this study raised the issue of whether a potential effect of the intervention was diluted by the heterogeneity of the sample population. In this regard, the latest anti-IL-5 antibody trials were performed on a small subset of individuals with asthma who had persistent sputum eosinophilia despite steroid treatment. Patients exhibited a significant reduction in exacerbations, prednisone sparing, and asthma quality of life questionnaire scores [61,64]. Blood and sputum eosinophil levels were, expectedly, significantly decreased, although conventional parameters of disease progression similar to those used in earlier studies again remained unchanged following treatment [61,64]. Ultimately, whether the reduction in exacerbations in this select set of asthmatic individuals is directly the result of eosinophil depletion or of the effects of IL-5 on other cells such as B cells, basophils, and ASM cells remains unsolved.

Given the interplay of IL-3, IL-5, and GM-CSF in contributing to the allergic phenotype, Sun *et al.* [66] generated a monoclonal antibody, BION-1, against a major cytokine-binding domain of βc. The antagonism of all three cytokines *in vitro* was shown to greatly reduce human eosinophil production, survival, and activation but its efficacy *in vivo* is still unknown. In this regard, Gauvreau *et al.* [67] showed that inhaled TPI ASM8 (Topigen Pharmaceuticals Inc., now part of Pharmaxis Ltd., French Forest, NSW, Australia), a mix of antisense oligonucleotides that downregulate βc and CCR3 mRNA, can attenuate allergen-induced eosinophilia and physiologic responses in individuals with mild allergic asthma.

Concluding remarks

It is clear that, in addition to their roles in emergency hematopoiesis, IL-3, IL-5, and GM-CSF regulate a vast array of activities on APCs, lymphocytes, mast cells, and granulocytes. The evidence indicates that these cytokines play a variety of roles in conditions as diverse as infections, cancer, autoimmunity, neurologic disorders, and allergic inflammation. In regards to the later, IL-3, IL-5, and GM-CSF are undoubtedly involved in the pathogenesis of allergic asthma, contributing to both disease development and progression and having, directly or indirectly, a major impact on the extent of inflammation, airway remodeling, dysfunction, and, ultimately, disease severity. However, the potential of these molecules to become significant therapeutic targets in allergic asthma is, in our view, limited for several reasons.

First, these molecules ontogenically evolved to become elements of the host defense program. Therefore, the potential benefits of neutralizing these molecules or inhibiting their effects must be assessed against the risk of compromising not only host defense but also other homeostatic activities, both in the short and long term. Second, as we have discussed earlier, there is a notable degree of redundancy and, consequently, the blockade of one single cytokine is likely to have modest effects on particular biologic activities. Moreover, there are additional layers of redundancy outside this triad of cytokines. For example, osteopontin and thymic stromal lymphopoietin may have some activities on APCs that are redundant with GM-CSF. Third, asthma is a complex and inherently heterogeneous disease. This implies that the relationships between a specific molecule and a particular biologic activity and, most importantly, a given functional, structural, or clinical outcome are nonlinear. The failure of anti-IL-5 trials in asthma "at large" and, particularly, the disconnection between decreases in eosinophils and unaltered functional parameters in a selected and rare group of steroid-resistant patients illustrates this point.

The development of future therapies for asthma faces an apparent dilemma: specific versus generic strategies. Specific strategies are confronted with the issues outlined above; generic strategies, of which corticosteroids are an archetype, are of limited benefit in patients with moderate or severe disease and must deal with the potential of detrimental adverse effects. However, the real challenge is to deconstruct the idea of asthma as a single or homogeneous disease. Indeed, the identification of distinct asthma phenotypes underlined by specific cellular and molecular pathways that extend or challenge current dogmas may be the only venue to discover new treatments for asthma.

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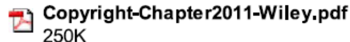

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