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Development of Immunologic Tolerance in a Murine Model of House Dust Mite-Induced Asthma

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Sonali J. Bracken, PhD

University of Connecticut, 2017

Allergic asthma is a leading cause of morbidity in industrialized nations and results from lack of immunologic tolerance to environmental antigens. Understanding the factors that contribute to immune tolerance is critical for the development of improved therapeutic strategies for asthma. Although much has been gained from studying ovalbumin (OVA)-induced mouse models of allergic airway disease (AAD), OVA is far less immunologically complex than most human-relevant antigens. House dust mite (HDM) is the most common cause of asthma worldwide. In a novel murine model of AAD, short-term (2 weeks) and intermediate-term (5 weeks) intranasal HDM exposure resulted in classic hallmarks of asthma, including airway eosinophilia and airway hyper-reactivity (AHR). Intriguingly, long-term (11 weeks) HDM instillation resulted in suppression of Th2-mediated disease processes, providing the first evidence of its kind that immunologic tolerance can develop with continuous exposure to a human-relevant allergen. Moreover, HDM-induced tolerance resulted in *suppression* of disease despite persistent lung inflammation rather than complete *resolution* of inflammation, which may be more clinically representative of the processes involved with tolerance development in humans. While long-term exposure to both OVA and HDM was associated with increased numbers of local $Foxp3^+$ regulatory T cells (Tregs), only long-term HDM exposure resulted in upregulation of IL-10 production by lung alveolar macrophages (AM). These AMs were found to be capable of inducing Tregs, suggesting that they may play an important role in the suppression of HDM-induced asthma. We believe that this macrophage population may be exploitable for the development of enhanced immunotherapeutic strategies for asthma.

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Development of Immunologic Tolerance in a Murine Model of House Dust Mite-Induced Asthma

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"Success consists of going from failure to failure without loss of enthusiasm."

- Sir Winston Churchill

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CHAPTER 1

INTRODUCTION

I. Allergic asthma: a global issue of epidemic proportion

"He was just starting to recover,

to recognize his comrades round him. He'd stopped

gasping and sweating, for aegis-bearing Zeus had revived his mind."

- *The Iliad, book XV, line 290*

Descriptions of the condition known as asthma have been floating around since the eighth century B.C., when the term was first used in reference to Hector's attempt to catch his breath post battle. The word 'asthma' has evolved quite a bit since it was originally described by Homer, who simply meant it to refer to the symptom of dyspnea (difficulty breathing) rather than the disease. Yet it is no wonder that the condition we know as modern-day asthma is rooted in the Greek language, having stemmed from the verb 'aazein' (*to pant or exhale with an open mouth*).

Asthma is a chronic inflammatory disorder of the airways that is characterized by recurrent symptoms of coughing, wheezing, dyspnea, and chest tightness. It occurs in people of all ages, races, and sexes in every nation in the world. Diagnosis is typically based on a mixture of history and clinical features (respiratory symptoms) with support from objective findings, including evidence of reversible airflow

obstruction (i.e. positive responses to bronchodilators). Nevertheless, definitions of asthma type and severity can be quite variable, as no gold standard test is currently available to confirm or rule out the diagnosis.

a. Epidemiology

Although asthma can come in many forms, the most common type is allergic asthma, accounting for greater than 60% of all cases*. The prevalence of asthma is extremely high in the Western world. Approximately 8% of individuals in the United States are afflicted by this condition, though frequency may be higher in specific demographic populations (smokers, urbanites, non-Hispanic blacks, children, etc.)[1]. Prevalence of asthma across other nations varies widely, but this gap is indeed narrowing due to rising prevalence in low and middle income countries. It is currently estimated that 300 million people worldwide suffer from this condition. In addition, a quarter of a million deaths occur annually as a direct result of asthma, many of which are entirely avoidable. Yet the biggest concern is that the incidence of asthma, which is already high, continues to grow year by year. The total number of people in the United States with asthma has increased by 2.9% annually, from 20.3 million (one in fourteen people) in 2001 to 25.7 million (one in twelve people; 27% children) in 2010[2]. By 2025, it is estimated that the number of asthmatics worldwide will increase by 100 million people[1]. It is clear that asthma is a global health problem that will soon reach epidemic proportions.

b. Economic and social burden

Although risk of premature death from asthma is relatively low, asthma is associated with tremendous morbidity. It imposes a high frequency of health care utilization and lost productivity at work

*The term 'asthma' will be used to refer to 'allergic asthma' throughout the remainder of the text

or school. A recent report suggested that approximately half of all individuals with asthma suffer from at least one asthma attack[†] annually. As a result, asthma is a major cause of hospitalization in adults and the third leading cause of hospitalization in persons under 18 years of age[3]. Asthma attacks send 1.8 million victims to the emergency room annually[4] and generate 50% of the approximately $60 billion annual heath care cost for asthma. Asthma also accounts for roughly 1% (14.2 million) of all annual ambulatory visits in the United States. It has gained a reputation for being one of the leading causes of work and school absenteeism and only continues to get worse. Unfortunately, the diagnosis of asthma is so common that these repercussions have been both accepted and trivialized by the general public.

If there is any doubt regarding the severity of the "asthma epidemic", one need only consider the heavy economic burden of this disease. In 2009, annual expenditures per asthma patient totaled $3,300 in direct health care costs and indirect expenses (i.e. lost productivity from missed school and work days)[5]. On the whole, yearly direct medical and indirect costs of asthma are estimated to be about $56 billion, accounting for over 1% of the annual $3.8 trillion health care expenditure. The often chronic nature of asthma further contributes to the tremendous economic consequences of this disease. Unfortunately, the increasing prevalence of asthma and rising costs of health care signify that this problem could become much worse without proper intervention.

II. Pathophysiology and mechanisms of disease

a. The intolerant immune system

In order to understand the causes of asthma, one must first acknowledge the key function of the immune system, which serves to protect the host against pathogenic invaders (bacteria, viruses, etc.)

[†] Potentially life-threatening situation in which the airways become swollen, inflamed, and constricted (i.e. reversibly obstructed)

through a variety of defense mechanisms we have loosely termed '*inflammation'*. Inflammation describes a complex biological response to harmful stimuli that involves a specialized set of cells (leukocytes) and molecular mediators. While inflammation is extremely effective at ridding the body of harmful organisms, it often takes casualties in the form of damage to host cells and tissues, thus rendering it undesirable unless absolutely necessary. In an effort to prevent immune reactions against non-threatening substances, the immune system has adopted a rather unique set of mechanisms to discriminate against self and non-self, a concept referred to as *immunologic tolerance*. Immunologic tolerance, which describes the state of non-reactivity or unresponsiveness against an antigen (Ag), is an extremely important phenomenon for maintaining homeostasis. Not only does it hinder the immune system from reacting against self Ags, it is particularly important for preventing reactions against environmental Ags, which are both ubiquitous and innocuous to the body. *The key players involved in tolerance will be further discussed in section IId and IIe*.

Although every individual is exposed to a ubiquitous array of allergens over the course of his/her lifetime, only a relatively small percentage proceed to develop allergic reactions. *Atopy* refers to the predisposition towards development of exaggerated immune responses against innocuous, environmental allergens. Thus, the pathophysiology of asthma and other atopic disorders may be best accepted as an intrinsic immune defect that manifests when exposed to specific environmental conditions. In the same manner that reactions against self Ags can lead to the development of autoimmune disease, lack of tolerance against environmental Ags (e.g. house dust mite [HDM], pet dander, mold, smoke) precipitates development of atopic disorders. Atopic reactions in the lung are responsible for symptoms of allergic asthma.

In light of this concept, several hypotheses have been proposed to account for this rising incidence of asthma, many of which relate to negative changes within our global environment (e.g. enhanced air pollution) over the past few decades. However, the best-known theory is, perhaps, the "hygiene hypothesis", which attributes increased asthma incidence (as well as incidence of other atopic disorders) to a variety of lifestyle changes that have reduced infection rates in developed nations[6]. Prolonged exposure to bacterial

products has been shown to be protective against development of asthma and atopy through chronic stimulation of Toll-like receptors (TLRs) on innate immune cells. Although the specific immunological mechanism that explains why reduced TLR stimulation during early childhood results in a heightened immune responses to allergens is still a matter of controversy, some have suggested that lower microbial burden reduces the activity of the regulatory immune cells that mediate proper tolerance development.

b. Type I hypersensitivity reactions: early-phase response to allergens and production of IgE

The type I hypersensitivity response is believed to underlie the majority of allergic reactions, including asthma. These responses are typically initiated upon sensitization to an allergen in a susceptible individual.

Sensitization phase

Airborne allergens are typically small yet complex proteins that are carried on dry particles (e.g. pollen, dust mite feces). When these particles make contact with the upper respiratory tract and/or airways, allergenic peptides are eluted off their carrier compounds and then diffuse into the mucosa. This action is facilitated by the proteolytic activity of many human allergens, which damages tight junctions of epithelial cells lining the airway and promotes barrier dysfunction[7]. In response to TLR signaling from allergens (e.g. HDM signaling of TLR4 on epithelial cells)[8], damaged epithelial cells release interleukin-25 (IL-25), IL-33, and thymic stromal lymphopoeitin (TSLP), the latter of which is crucial for the activation of dendritic cells (DCs) and aids in programming DCs to become Type 2 helper (Th2) cell inducers[9,10]. IL-25 and IL-33, on the other hand, are critical for the activation of type 2 innate lymphoid cells (ILC2), which immediately respond by secreting a host of Th2 cytokines. In addition, IL-25 stimulates the accumulation of type 2 multipotent progenitor (MPP^{type2}) cells in the mucosa that give rise to cells of the monocyte-macrophage and granulocyte lineages capable of promoting type 2

inflammation[11]. These actions ensure an immediate response to epithelial cell damage even prior to the activation of Th2 cells.

Following damage to the epithelial surface, allergens gain access to an extensive submucosal dendritic cell (DC) network. In addition, β-glucan-rich motifs on specific allergens (e.g. HDM) can interact with dectin-1 on bronchial epithelial cells and stimulate their production of CCL20, a chemokine that is vital for attraction of immature lung DCs[12]. Myeloid DCs pick up and process the Ag, after which time they undergo an extensive maturation process. During this time, DCs lose their intrinsic ability to process Ag but acquire full Ag-presentation capacity (e.g. expression of co-stimulatory molecules and T cell chemokines)[13]. MHCII-loaded DCs then migrate from the mucosa to the T-cell rich areas of the hilar lymph node (HLN)*, where they await the arrival of Ag-specific T cells. Upon encountering these cells, DCs subsequently prime them towards a Th2 phenotype via expression of Th2-instructive cell surface signals (e.g. Notch ligand jagged-2, c-Kit, OX40L)[14]. However, it is important to note that DCs have not been found to be an important source of IL-4, the hallmark Th2 polarizing cytokine. Interestingly, it has been shown that basophils are an important source of IL-4 and may serve as a *bona fide* APCs that can provide peptide-MHC, costimulation, and Th2-polarizing cytokines even in the absence of DCs[15,16]. As a result, many have speculated that DCs may have a redundant role in the process of allergen sensitization; however, their necessity in priming Th2 cell responses is still hotly debated.

Th2 cells can influence B cell class switching to IgE-secreting plasma cells. This process requires two signals: 1) STAT-6-mediated transcription at the IgE isotype-specific switch region (influenced by IL-4 or IL-13) and 2) ligation of CD40 on B cells with CD40L on T cells. At the initiation of class switching, T cells provide both signals; however, basophils have been suggested to play a role in polyclonal amplification of IgE[17]. Following initiation of its production, IgE then binds to the surface of mast cells, basophils, and eosinophils via interactions of its constant region with the high-affinity FcεR1. At this point, the sensitization phase has been completed.

*Lung-draining lymph node

c. Disease progression:

Allergen challenge: early phase

Once the triggering allergen is re-introduced to the host, it is bound by IgE on the surface of mast cells, which leads to cross-linking of FcεRI. This cross-linking results in subsequent recruitment of Syk kinase, which stimulates a number of downstream signaling events associated with activation of mast cells, basophils, and eosinophils. Mast cells, in particular, may release pre-formed mediators such as histamine, which allows for rapid initiation of an inflammatory reaction in response to Fc receptor triggering[18]. Histamine is a major contributor to the phenotype associated with allergic asthma. Not only can it bind to H1 receptors on airway smooth muscle cells and cause bronchoconstriction, it also stimulates widespread vasodilation, leading to mucosal edema. In addition, mast cells can rapidly synthesize molecules such as phospholipids (e.g. prostaglandins and leukotrienes), the latter of which can promote bronchoconstriction, enhanced permeability, and chemotaxis of neutrophils. Mast cells are also involved in the production of chemokines that recruit T cells (RANTES, TARC), macrophages (MCP-1), and eosinophils (eotaxin) to the lung tissue. Mast cell and basophil degranulation in response to crosslinking of the IgE-FcεRI complexes by allergen constitutes the *immediate phase* of the allergic reaction.

Late phase

IgE may also bind to FcεRI on the surface of DCs and monocytes as well as to the low-affinity IgE receptor (FcεRII; CD23) on the surface of B cells. This process facilitates allergen uptake by these APCs, where they are subsequently presented to allergen-specific $CD4^+$ T cells. $CD4^+$ T cells drive the *late phase* of the allergic reaction. In response to subsequent allergen exposure, newly polarized Th2 cells respond by secreting cytokines and chemokines that augment the allergic response. These include molecules such as IL-4, IL-13 and IL-5, all of which have been shown to be increased in the BAL and

serum of allergic individuals[19]. IL-4 is, perhaps, the most classic and important Th2 cytokine in the development of asthma. One of its essential biological activities involves the Stat-6-mediated conversion of naïve T cells into Th2 effector cells that are capable of initiating asthmatic responses. IL-4 is also a critical player in the process of B cell immunoglobulin isotype switching from the IgM subclass to IgE, another important function in the pathogenesis of asthma. Aberrant production of IL-4 or hyperresponsiveness to this cytokine may contribute to the pathology of this disease; asthma has indeed been genetically linked to polymorphisms in the genes encoding the IL-4 receptor, Stat-6, and BCL6, the latter of which counteracts the effects of Stat-6[20]. Studies in mice have demonstrated that neutralizing the effects of IL-4 before allergen sensitization inhibits the development of allergen-specific IgE and attenuates eosophil influx in an IL-5-dependent manner[21]. Interestingly, the same treatment given after priming but before allergen challenge was not effective at preventing allergic inflammation. Thus, while IL-4 is likely important for committing cells to the Th2 lineage, it is not as essential as other Th2 cytokines for the effector phase of the asthmatic response.

Because of its structural similarity to IL-4 and sharing of receptor components, IL-13 was hypothesized to play a role in the development of allergic responses. Studies have demonstrated that IL-13 is consistently overexpressed in the lungs of human asthmatics and that polymorphisms in this gene can contribute to susceptibility and pathogenesis of asthma[22]. Unlike IL-4, IL-13 does not have a role in the differentiation of Th2 cells; however, it has been shown to be important for the effector arm of the allergic immune response. When IL-13 but not IL-4 was blocked in allergen-challenged mice via administration of a soluble form of the IL-13Rα2 chain, results demonstrated a reversal of mucus production and airway hyperreactivity (AHR), two hallmark features of asthma that lead to cardinal signs of wheezing and dyspnea[23,24]. Airway plugging through hypersecretion of mucus is one of the primary reasons for dyspnea in asthmatic patients (i.e. due to airway obstruction). The other reason can be attributed to the hyperresponsiveness of smooth muscle to bronchoconstricting agents, leading to the phenomenon of AHR (increased sensitivity of the airways to the phenomenon of bronchospasm upon

interaction with an inhaled constrictor agonist). Further evidence of IL-13's role in asthma was demonstrated when acute administration of this cytokine was sufficient to produce AHR, eosinophilia, and mucus cell hyperplasia in naïve and RAG-deficient animals[23,24]. However, despite the importance of IL-13 in the effector phase of the asthmatic response, the exact mechanisms by which it induces AHR and mucus hypersecretion remain unclear. One mechanism that may help to explain the precise role of IL-13 in disease pathogenesis is its ability to induce a host of chemokines that can selectively recruit various leukocyte subtypes into the airways[23]. IL-5 is critical for the regulation of eosinophil development, proliferation, survival, and activation[25]. Transgenic mice expressing constitutive IL-5 in all T cells demonstrate severe and chronic eosinophilia, which can be observed in both the lungs and HLN[26].

In addition to Th2 cells and mast cells, eosinophils have been classically considered to be a hallmark cell of asthma. Under homeostatic conditions, eosinophils develop from $CD34^+$ progenitors in the bone marrow under the influence of IL-5, a cytokine which, as mentioned above, helps to promote their activation and survival. Eosinophils then migrate from the bone marrow into the blood (1-3% of blood leukocytes), and subsequently circulate through the lymphoid tissue and lung in low numbers. In response to allergen challenge, a significantly greater proportion of eosinophils are recruited to the lung under the influence of the chemokines produced by the airway epithelium, chemokine ligand 11 (CCL11; eotaxin-1) and CCL2 (eotaxin-2), as well as the Th2 cytokine IL-13[27]. Once in the lung, eosinophils execute a variety of functions, including secretion of preformed mediators (e.g. major basic protein, eosinophil cationic protein, eosinophil peroxidase) that cause damage to the airways, synthesis and secretion of leukotrienes, cytokine and chemokine production, and exacerbation of Th2 responses. Studies in eosinophil-deficient PHIL mice exposed to OVA Ag have shown that AAD-associated histopathology and AHR was reduced when compared to wild type (WT) mice[28], suggesting that eosinophils have a central role in the pathophysiology of asthma. Furthermore, intratracheal transfer of eosinophils into OVA-treated $IL\text{-}5^{-/-}$ mice (in which eosinophils are severely lacking) resulted in restoration of mucus hypersecretion, BAL Th2 cytokine levels, and AHR[29]. Recent studies from animal models of asthma have

demonstrated that eosinophils may also play a significant role in the enhancement of Th2 responses by acting as professional antigen presenting cells (APCs). After exposure to allergen, eosinophils upregulate MHCII and levels of co-stimulatory molecules (CD40, CD80, CD86) and migrate to the local draining lymph node of the lung, where they are capable of priming T cell responses[30].

Note: Many additional cell types beyond Th2 cells and eosinophils are implicated in the pathogenesis of asthma, including macrophages (*discussed in Section IIe*), DCs, lymphocytes, epithelial cells, NK cells, neutrophils (particularly in severe asthma), and innate lymphoid cells. Although the contributions of these cell types will not be specifically discussed in detail within this dissertation, the tremendous complexity of asthma (and the hurdles we face to cure it; *see Section III*) are reflected in the myriad of cell types involved in this disease and their overlapping functions.

Chronic disease

As a result of chronic inflammation in the lung, bronchial airways undergo significant remodeling over time. Airway remodeling is a term that indicates changes to the composition, quantity, and organization of the cellular components of the airway wall due to chronic injury and repair. These structural alterations can contribute to the loss of lung function that is commonly seen in asthmatic patients, and include airway wall thickening, epithelial injury/shedding, airway smooth muscle hyperplasia/hypertrophy, goblet cell hyperplasia, and subepithelial fibrosis[31]. While increases in airway smooth muscle mass and mucus hypersecretion can both exacerbate the airway obstruction and narrowing that are classically associated with asthma, the pathophysiologic consequences of subepithelial fibrosis are less clear. However, this process is typically irreversible and tends to correlate with the decline in FEV1 that is characteristic of asthma[32].

Unfortunately, the stimuli that contribute to airway remodeling are not well defined due to the limited availability of lung biopsy specimens from patients with chronic asthma. However, TGF-β is thought to have a substantial role in this process. TGF-β is produced by fibroblasts, eosinophils, macrophages, and lymphocytes and is detected in high quantities in the BAL of asthmatic patients relative to control

subjects[33]. Its levels often correlate with basement membrane thickness and overall disease severity. Other cytokines that may be involved in the process of subepithelial fibrosis include GM-CSF[34] and IL-13[35], the latter of which has been associated with upregulation of arginase-1[36]. Some studies have demonstrated that remodeling is unchanged or only mildly attenuated by the presence of steroid therapy[37]. Curative therapies for asthma are required to prevent this deterioration in lung function over time.

d. Restoring homeostasis in the airways: The critical role of Regulatory T cells (Tregs)

Albeit a powerful tool for ridding the body of harmful pathogens, the immune system must refrain from mounting reactions against self proteins and innocuous Ags, lest it unnecessarily damage host cells and tissues (*see Section IIa*). This requires that the immune system have a regulatory arm to restrain itself in the face of excessive inflammation. Of regulatory immune cells, none is more widely regarded or studied than the regulatory T cell (Treg).

Discovery of Tregs

There is no doubt that the body has developed several important mechanisms for preventing reactions against non-pathogenic Ags. Negative selection of self-reactive T cells in the thymus leads to elimination of high-affinity T cell clones that recognize self, and engagement of TCRs by self proteins can induce a state of anergy (unresponsiveness). Moreover, the two-signal hypothesis of T cell activation requires that APCs deliver adequate co-stimulatory signals to T cells in addition to stimulation of the TCR, many of which do not arise in the absence of strong inflammatory signals. Nevertheless, these (central and peripheral) mechanisms of tolerance have been insufficient to counteract the threat of immune-mediated pathology without the presence of a specialized subset of T cells acting in conjunction to dampen the immune system. Initial evidence in support of the development of natural (thymic-generated) T cells capable of performing this function came from early thymectomy experiments, which effectively demonstrated that thymic removal between d2 and d4 of life resulted in T cell-mediated tissue

damage[38,39]. However, this pathophysiological process was discouraged by the transfer of thymocytes or splenocytes from euthymic adult mice, suggesting that a population of T cells generated in the thymus around d3 of life is essential for prevention of autoimmune disease[40,41]. This early work culminated in the eventual discovery of a $CD4^+$ T cell subset expressing high amounts of CD25 (high-affinity IL-2 receptor α-chain). These cells, termed Tregs, were proven to have a unique suppressive potential[42]. Unfortunately, the utility of identifying Tregs on the basis of CD25 expression was limited by the fact that all activated T cells upregulate this marker.

More insight into the biology of Tregs were facilitated by studies in mice with mutations in the forkhead box P3 (Foxp3) transcription factor and in humans with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. It appeared that both sets of subjects were afflicted with a deadly, early-onset disorder involving both autoimmune and allergic components[43]. On the basis of these observations, murine studies were conducted that revealed stable expression of Foxp3 in naïve $CD4^+CD25^+$ T cells (Tregs) but not in naïve $CD4^+CD25^-$ cells or in activated T cells[44]. Furthermore, these cells were also shown to confer suppressor function on peripheral T cells[45]. Since this time, many additional papers have demonstrated that Foxp3 is critical for Treg differentiation* and suppressor function. It is also the most specific and widely accepted marker of the Treg lineage, particularly in mice†.

In addition to natural (thymic-derived) Tregs (nTregs), $Foxp3^+$ Tregs can also be generated in the periphery; these are known as inducible or adaptive Tregs (iTregs)[46]. Other inducible Treg subtypes include $Foxp3^-IL\text{-}10^+$ TGF-β^+ Tr1 cells and $Foxp3^-$TGF-β^+ Th3 cells. In addition to $CD4^+$ T cells, $CD8^+CD28^-Foxp3^+$ T suppressor cells have also been described. The functions of Tr1 cells will be discussed more in *Section III;* however, the remainder of this discussion will specifically focus on mechanisms of $Foxp3^+$ Tregs. In addition to their origin, $Foxp3^+$ nTreg and iTreg subpopulation differ in their phenotype, differentiation requirements (the former requires recombination-activating gene [RAG]),

* In addition to Foxp3, IL-2 and TGF-β (and to a lesser extent, IL-7 and IL-15) are critical for Treg differentiation
† In humans, Foxp3 is a less specific Treg marker as it can also be expressed on activated T cells

and homing behavior[47]. It has also been reported that the two may have differential TCR repertoires; nTregs may be more important for controlling autoantigen reactions (thus preventing autoimmunity), while iTregs are more commonly generated in response to innocuous, environmental Ags[46]. However, this opinion is rather controversial.

Importance of Tregs in asthma

In addition to prevention of autoimmune disorders, the phenotype of patients with IPEX syndrome demonstrates that Tregs also play a critical role in the regulation of asthma and other allergic diseases. Decreased Treg levels or defective Treg function has been associated with development of hyper IgE syndrome, hypereosinophilia, and allergic airway inflammation in Foxp3 mutant mice[48]. It has been theorized that the reason why only a select percentage of individuals develop asthma is because their suppressive immune cells (i.e. Tregs) are either 1) decreased 2) defective or 3) overwhelmed[49]. Patients with asthma who lack this tolerance have been shown to have decreased Tregs in their airways[49,50], a reduced ratio of Tregs to IL-4-secreting T cells, as well as decreased expression of Foxp3 in $CD4^+CD25^{hi}$ T cells[51]. In addition, studies have shown that $CD4^+CD25^+$ T cells from non-atopic donors suppress proliferation and IL-5 production by their own, allergen-stimulated $CD4^+CD25^-$ T cells to a greater degree than $CD4^+CD25^+$ T cells from atopic donors, the latter of which show better suppressive capacity than $CD4^+CD25^+$ T cells isolated from patients with hay fever during the pollen season[52]. This demonstrates that Tregs from atopic and allergic individuals are indeed defective in some capacity. A similar reduction in Foxp3 expression and Treg suppressive capacity was demonstrated in HDM-allergic children compared to healthy controls, which was restored when anti-TNF-α was administered[51]. Interestingly, Hartl et al. demonstrated that the frequency of Tregs and Foxp3 mRNA in the BAL correlate positively with FEV1 in children with asthma, suggesting that Tregs (or a lack thereof) may play a critical role in reduction of pulmonary function observed in asthmatic individuals (an effect that may be influenced by their suppression of Th2 cytokines and chemokines)[53]. Moreover, successful treatment of asthma with

corticosteroids (*see Section IIIa*) and allergen-specific immunotherapy (*see Section IIIc*) have both been associated with increases in Tregs and improvements in inflammation and pulmonary function[54].

The pivotal role of Tregs in maintaining immune tolerance within the airways has also been demonstrated in animal models, in which intravenous (i.v.) adoptive transfer of Ag-specific Tregs to OVA-sensitized mice was shown to prevent development of allergic inflammation and AHR after OVA challenge and increase lung expression of IL-10[55]. This immunoregulation was essentially reversed when an anti-IL-10R antibody was administered but not when Tregs from IL-$10^{-/-}$ mice were transferred, suggesting that Tregs suppress via an IL-10-dependent mechanism but do not need to make it themselves. Follow-up studies revealed that when delivered after disease onset, Tregs were also capable of downregulating established inflammation and hindering airway remodeling[56]. Moreover, depletion of Tregs in AHR-"resistant" C3H mice prior to the administration of HDM led to exacerbated airway inflammation and AHR that was associated with an increase in the number of myeloid DCs, expression of MHCII and co-stimulatory molecules on these DCs, as well as an enhanced ability of DCs to stimulate T cell proliferation and Th2 skewing[57]. These data suggest that Tregs are extremely important in preventing allergen sensitization through inhibitory effects on DC activation. Interestingly, Leech et al. showed that naïve T cells are capable of exerting suppressive effects when transferred into Der p 1-challenged mice[58], suggesting that Tregs may not be Ag specific to suppress airway disease (though this is hotly debated). Methods to expand Tregs, recruit them to the site of inflammation, and enhance their suppressive potential has been the focus of many recent studies and appears to be of promising therapeutic value in patients with asthma[59,60].

Mechanisms of Treg suppression in asthma

Tregs utilize multiple suppressor functions to regulate allergic inflammation, including inhibitory cytokine secretion, cell-contact dependent mechanisms, consumption of limiting growth factors, and modulation of DC activity[61]. A few of the mechanisms will be briefly discussed in the context of asthma.

Of inhibitory cytokines, IL-10 is among the most critical in the context of asthma. IL-10 is a potent inhibitor of Th2 cells, APCs, and proinflammatory cytokine production[62]. IL-10 also increases the ratio of IgG4 to IgE (*discussed more in section IIIc*). Studies in asthmatic patients have shown decreased levels of IL-10 in the BAL when compared to healthy controls[63], while children who suffer from asthma have been shown to have decreased IL-10 mRNA levels in T cells than in healthy children[64]. Moreover, IL-10 has been shown to be required for functional activation of Tregs[65]. TGF-β is also an important mediator in allergic inflammation. TGF-β suppresses the activity of both Th1 and Th2 cells and downregulates the expression of FcεRI on mast cells[66]. It inhibits IgE production while promoting class switching to IgA[67], which is beneficial for reducing allergic inflammation[62]. TGF-β also upregulates the expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4; discussed later) on the surface of T cells. However, the role of TGF-β in allergic inflammation is somewhat controversial, as this cytokine may also contribute to airway remodeling[62].

The question of whether IL-10 and TGF-β require cell-cell contact for Treg-mediated suppression in the context of asthma has been explored by Joetham et al, who demonstrated that direct intratracheal instillation of rIL-10 and rTGF-β reduced AHR in OVA-challenged mice to baseline levels, whereas neither cytokine on its own was capable of this function[65]. These cytokines also exerted synergistic effects in reducing BAL eosinophilia and Th2 inflammatory mediators, suggesting that cell-mediated contact is not required for the anti-inflammatory actions of IL-10 and TGF-β. However, one of the major mechanisms by which Tregs inhibit effector T cells (Teff) is through cell contact-dependent inhibition of proliferation[68]. Tregs also downregulate Th2 differentiation by inhibiting transcription factors such as Gfi-1, which otherwise help to promote Th2 expansion[69]. Others have shown that Tregs also have an indirect role in regulating Th2 responses through expression of Itch, an E3 ubiquitin ligase. Itch plays a major role in restraining Tregs from producing Th2 cytokines that, in turn, feed Th2 effector differentiation in non-Tregs[70].

Furthermore, Tregs modulate the activity of DCs and other APCs by conditioning them to produce immunosuppressive molecules and downregulating their capacity to activate Teff cells. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is expressed by Tregs and other T cells upon activation and can downregulate expression of co-stimulatory molecules (e.g. CD80 and CD86) on DCs via direct competition with CD28[49]. Thus, Tregs compete with naïve T cells for access to DC co-stimulatory signals and in turn, reduce their ability to activate Teff cells. Indeed, studies have demonstrated that the frequency of CTLA-4$^+$ Tregs in the sputum of asthmatic patients is significantly reduced when compared to healthy controls[71]. CTLA-4-induced signaling can also promote indoleamine 2,3-dioxygenase (IDO) activity and decreased glutathione synthesis in DCs, which leads to cell cycle arrest in T cells and further generation of iTregs[62]. By binding to MHCII, expression of LAG-3 on Tregs decreases DC activation and reduces their Ag presentation capacity. In addition, IL-10 suppresses the expression of costimulatory molecules and proinflammatory cytokines by DCs, which also promotes additional iTreg generation. Other suppressive actions of Tregs include inhibition of DC migration to lymph nodes in order to prevent priming of Teff responses.

Expression of CD39 and CD73 on Tregs has been shown to generate adenosine from ATP, ADP, and AMP. Adeosine can then engage A_{2A} receptors on Teff cells, which downregulates NF-κB and suppresses T cell responses[72]. CD39 also promotes the expression of Foxp3; decreased Foxp3 and CD39 mRNA have both been observed in patients with moderate to severe asthma when compared to patients with mild asthma, along with elevated levels of ROR-γt mRNA[73]. Thus, certain Tregs may act via CD39 to decrease severity of asthma by stabilizing expression of Foxp3 and preventing their conversion to Th17 cells. Other functions of Tregs include inhibitory effects on mast cells through cell-cell contact of OX40 (on Tregs) and OX40L (on mast cells), an action which ultimately reduces calcium influx into mast cells and prevents degranulation[74].

e. Good cop/bad cop? Alveolar macrophages and asthma

Macrophages: a heterogeneous and plastic group of cells

In 1908, Elie Metchnikoff received the Nobel Prize for his description of phagocytosis in macrophages. Since this critical discovery, immunologists have been obsessed with the role of macrophages in innate immune (i.e. non-specific) host defense. However, by primarily focusing on the microbicidal functions of macrophages, their major role in maintaining homeostasis has been largely ignored until recently.

Perhaps the most interesting feature of macrophages is their tremendous heterogeneity and plasticity. This allows them to effectively respond to environmental signals while preserving their role in maintaining immune homeostasis. As of recently, a great deal of effort has focused on subtyping macrophages into various classes that align with their primary effector functions. The concept of classic and alternative activation, or M1 and M2, was originally adopted to mimic the Th nomenclature. However, this classification system was soon found to be too generalized and limiting, and so the definitions of M1 and M2 have been expanded to incorporate three major functions: host defense, wound healing, and immunoregulation.

The term "classically activated" (M1) macrophage (CAM) was introduced in the 1960s to describe the non-specific but Ag-dependent microbicidal activity of macrophages towards Bacillus Calmette-Guerin and Listeria. These cells were later linked to Th1 responses; Th1 cells secrete INFγ, which activate classically activated macrophages and stimulate their production of pro-inflammatory cytokines (e.g. TNFα, IL-12) and free radicals[75]. CAMs are key mediators of the inflammatory reactions that occur in response to both extracellular and intracellular pathogens. They have enhanced phagocytic abilities and Ag presentation capacities[76]. In addition, they secrete matrix metalloproteinases, which are important for their migration through tissue during inflammatory responses. CAMs are thought to originate from classical $CD14^{hi}$ $CD16^{lo}$ monocytes ($Ly6c^{hi}$ monocytes in mice)[77].

Unlike CAMs, which are critical for host defense against infectious agents, M2 macrophages are considered to be more important for wound healing and immunoregulation. M2 macrophages are primarily divided into two subtypes: alternatively activated macrophages (AAM) and "regulatory" macrophages*. AAMs are wound-healing macrophages that play an important role in tissue remodeling. Like Th2 cells, they are also thought to provide protection against helminths[76]. IL-4 and IL-13 production during tissue injury rapidly polarizes resident macrophages into alternatively activated cells through the induction of STAT6, which leads to the upregulation of arginase, a pro-fibrotic marker by which these cells are commonly identified. In addition, these cells are characterized by enhanced expression of scavenger receptors for phagocytosis of debris (e.g. mannose receptor [CD206]) as well as chitinase-3-like-protein-3 (Chi3l3; also known as YM1) and resistin-like molecule α (Relmα; also known as FIZZ1).

IL-10$^+$ macrophages arise during late stages of the immune response in order to dampen inflammation[75]. They are generated by a number of stimuli (e.g. IL-10, glucocorticoids, prostaglandins, phagocytosis of apoptotic cells, immune complexes) that must act in concert with a TLR stimulus. As name implies, IL-10$^+$ macrophages produce high amounts of IL-10 and may also produce TGF-β (though this has not been confirmed because of the significant overlap between AAM and IL-10$^+$ macrophage markers[76]).

Despite the aforementioned classification system, it is important to note that macrophages are an extremely heterogeneous population of cells that are capable of adopting a range of phenotypes. In other words, these cells may exhibit characteristics of more than one major macrophage subtype to form so-called hybrid populations. Thus, macrophage phenotype and function should be appropriately considered as a spectrum rather than a strict classification system[75].

Pathogenic actions of M1 and M2 macrophages in asthma

∗ In 2014, a panel of macrophage experts proposed that the term "regulatory" macrophage be avoided because all macrophages are thought to have some degree of regulatory capacity[233]. Thus, "regulatory" macrophages will be simply referred to as IL-10$^+$ macrophages throughout this dissertation.

Although asthma is a Th2-dominant disease, both M1 and M2 macrophages have been implicated in the pathogenesis of this disorder. Levels of INFγ, an M1-inducing cytokine, have been shown to be elevated in asthmatic patients and correlate with severity of asthmatic inflammation[78]. INFγ has also been (indirectly) implicated in the development of AHR[79], as has TNFα[80], a product of M1 macrophages. In addition, TNFα has been shown to be involved in recruitment of eosinophils[81] and inhibition of $Foxp3^+$ Treg activity[82]. IL-6 and IL-1β, both of which are CAM products, have been shown to have direct effects on Th2 cell cytokine production[83], fibroblast activation[84], and granulocyte macrophage colony stimulating factor (GM-CSF) production[85], the latter of which enhances eosinophil recruitment and survival[86]. High levels of nitric oxide produced by CAMs have additionally been observed in asthmatic subjects[87] and may amplify lung injury and mucus production[88].

It is no surprise that AAMs are also highly abundant in asthmatic lungs, an effect that can be attributed to elevated levels of both IL-4 and IL-13. Indeed, it has been shown that asthmatic individuals have a higher number of AAMs on bronchial biopsy than healthy control subjects and that this correlates positively with declines in pulmonary function in these subjects[89]. Animal studies have demonstrated that female BALB/c mice, which tend to have more severe airway disease in response to the model Ag ovalbumin (OVA; *see section IVa-IVb*), have more AAMs in their lungs than their male counterparts[90]. Furthermore, artificially elevating AAM numbers in male mice via intratracheal instillation of these cells dramatically increased BAL eosinophilia, mDC migration to the lymph nodes, and T_{eff} to Treg ratio, suggesting a causal role for AAMs in severity of asthma (*Fig 1*).

Alveolar macrophages: potential suppressive actions in asthma

Of cell types that are critical for the maintenance of homeostasis in the lung, the alveolar macrophage (AM) is among the most important. AMs are one of two major macrophage subtypes found in the lung (the other being the interstitial macrophage; IM). They colonize the airways within the first few days after birth (a process dependent on fetal monocytes) and proliferate *in situ* to maintain a resident population of long-lived cells throughout life[91]. AMs are generally considered to be Siglec-

$F^{hi}F4/80^{+}CD11c^{+}$ cells, as opposed to IMs, which are of an $F4/80^{+}Siglec\text{-}F^{-}CD11b^{+}CD11c^{-}$ phenotype. They can adopt both M1 and/or M2 functional markers.

As resident cells of the conducting airways and alveolar spaces, AMs are among the first cells to encounter inhaled Ags. Because the airways are exposed to a never-ending barrage of microbial, viral, and environmental toxins, one of the principal roles of AMs involves initiation of inflammatory responses (via conversion to M1 cells) during times of need. However, excessive inflammation could potentially disrupt normal gas exchange, and so AMs must also be capable of suppressing unwanted immune responses. How these cells switch back and forth so rapidly between these two opposing functions is a subject of great interest in the field. It has been suggested that destruction of the airway epithelium and of regulatory ligands on this epithelium (e.g. CD200) may actually stimulate AMs to respond in a pro-inflammatory as opposed to regulatory manner[91]. In addition, pathogen-specific properties that may lead to upregulation of specific pattern recognition receptors (PRR) on the surface of AMs and subsequent signaling through these receptors may help to determine the overall fate of these cells in any given situation. As the focus of this dissertation involves regulatory aspects of AMs, this function will exclusively be considered throughout the remainder of the text. However, it is nevertheless important to note that AMs may be pro-inflammatory and potentially pathogenic in a variety of circumstances.

Several studies have supported a tolerogenic role for M2 AMs in response to allergen challenge, although their phenotypic classification into specific M2 subsets has not typically been reported. Careau et al. elegantly demonstrated that depletion of AMs using liposomes instilled with clodronate (Cl_2MDP) in allergen-resistant Sprague Dawley rats promoted methacholine-induced AHR post challenge with the model allergen OVA[92]. Moreover, adoptive transfer of AMs from Sprague Dawley rats into allergen-susceptible Brown Norway rats prior to allergen challenge abrogated AHR responses in a dose-dependent manner, suggesting that AMs may downregulate mast cells or other factors that promote bronchial responsiveness. Similar findings were confirmed in mice by Bang et al[93], who demonstrated a significant reduction in OVA-induced allergic airway inflammation when unsensitized AMs were intratracheally

transferred to AM-depleted, OVA-sensitized mice prior to allergen challenge. Depletion of AMs was also shown to exacerbate HDM-induced allergic inflammation, as noted by elevated eosinophils, neutrophils, Th2 cells, and Th2 cytokine and antibody responses[94]. Moreoever, depletion of AMs, which are critical for phagocytosis of apoptotic cells[95], also delayed resolution of the allergic response following discontinuation of the allergen. Other studies on AM function in HDM-induced allergic models have shown that depletion of AMs leads to enhanced recruitment of $Ly6c^{hi}CD11b^{+}$ monocytes to the lungs and exacerbation of inflammation, suggesting that while resident AMs are anti-inflammatory in the context of asthma, monocyte-derived cells are not[96]. These $Ly6c^{hi}$ monocytes may be potentially influenced towards a pathogenic AAM phenotype following their arrival to an AM-depleted lung environment where high levels of Th2 cytokines are present[97].

One of the major mechanisms by which AMs exert their anti-inflammatory actions is through suppression of T cell stimulation. Coleman et al. have demonstrated that while peritoneal (control) macrophages promote ^{3}H-thymidine incorporation by anti-CD3-stimulated naïve T cells, AMs fail to promote this incorporation[98]. This effect is likely due to suppressive AM-derived soluble factors because the same effects were noted when T cells were co-cultured with AM-derived culture media in lieu of actual cells. One of these major factors is IL-10. As mentioned above, $IL\text{-}10^{+}$ M2 macrophages exert an immunoregulatory role in a number of diseases, and they have been shown to inversely correlate with severity of asthma (Figure 1). Indeed, studies have demonstrated that lung macrophages produce less IL-10 in steroid-naïve asthmatic subjects than in healthy subjects[99] and significantly upregulate IL-10 post treatment with traditional pharmacologic agents[100], suggesting that defects in development of these cells may predispose individuals to development of this disease. $IL\text{-}10^{+}$ M2 AMs but not M1 CAMs or M2 AAMs have also been shown to be lower in the lungs of mice exposed intranasally to HDM relative to control animals, an effect that negatively correlated with disease parameters[101]. IL-10-expressing M2 macrophages demonstrate enhanced indoleamine 2,3-dioxygenase (IDO) activity, which may subsequently reduce Th2-mediated inflammation in asthma[100]. Additional findings suggest that $IL\text{-}10^{+}$

macrophages (IMs, in this case) may downregulate allergic inflammation and AHR by suppressing LPS-stimulated DC secretion of pro-inflammatory cytokines as well as DC maturation and migration to lymph nodes, where Ag presentation would otherwise occur[102]. IL-27 is another cytokine that may be important for the regulation of inflammatory responses by AMs[94].

Effects of AMs on induction of Tregs

Recent studies have suggested that one of the major mechanisms by which M2 AMs may exert anti-inflammatory effects under homeostatic conditions is through the induction of $Foxp3^+$ Treg cells, an action that appears to be dependent on both TGF-β and retinoic acid[98,103]. Moreover, this conversion was still possible even when T cells were first activated in a short-term manner by Ag-bearing lymph node DCs[103]. Since AMs are not found in high frequency in the HLN, this suggests that they may act as both secondary and tolerogenic APCs within the lung. Interestingly enough, intratracheal transfer of Ag-pulsed AMs prior to OVA sensitization have also been shown to protect against development of allergic airway inflammation, as is evidenced by decreased lung inflammation, Th2 cytokine production, BAL total leukocytes, eosinophil levels, and AHR as well as increased pulmonary Tregs[103]. Other studies have demonstrated that inhibiting macrophage-derived retinoic acid *in vivo* subsequently promotes enhanced IgE titers when mice are first sensitized and challenged with OVA-CpG[98], an effect that may also be dependent on their induction of $Foxp3^+$ Tregs. However, inhalation of complex allergens such as HDM have been shown to impair AM ability to induce $Foxp3^+$ Tregs due to stimulation of a pro-inflammatory phenotype despite lack of effect on TGF-β or retinoic acid production[103]. Furthermore, production of $IL\text{-}10^+$ "regulatory" M2 AMs with prolonged exposure to a human-relevant allergen (e.g. HDM) has, to the best of our knowledge, never before been demonstrated.

Of note, the specific role of IL-10 in induction of $Foxp3^+$ Tregs has not been explored in great detail. In an elegant series of experiments, Denning et al. demonstrated that lamina propria M2 macrophages are capable of inducing $Foxp3^+$ Tregs in an IL-10-dependent manner[104]. Others have shown that IL-10-

producing macrophages are more critical for the maintenance of Foxp3 expression in the presence of inflammation[105].

III. Why has asthma gone uncured?

"The young physician starts life with 20 drugs for each disease, and the old physician ends life with one drug for 20 diseases."

- *William Osler*

a. Pharmacologic treatments for allergic asthma

Although science and medicine are advancing at a rapid pace, the stunning reality is that the majority of human diseases are still lacking for cures. Asthma is among this list of diseases. Of the roughly $56 billion being spent on asthma in the United States every year, over $6 billion is being funneled towards the costs of prescription drugs. Unfortunately, current pharmacologic agents for asthma are primarily directed at symptom relief and have limited curative potential and poor prospects for reducing long-term disease prevalence.

While there is no cure for asthma, it can indeed be controlled with appropriate medical care, and routine exacerbations can be minimized by avoiding exposures to the triggering allergen. As a result, mortality risk associated with asthma in developed countries is quite low. In the early days, asthma was viewed as a disease of bronchoconstriction, and bronchodilators were the mainstay of treatment. Bronchodilators are important for relief of asthma symptoms and work through their direct relaxation effects on airway smooth muscle cells. There are three major classes of bronchodilators: β2-adrenoceptor agonists, muscarinic receptor antagonists, and xanthines. Fast-acting agents (e.g. albuterol) have become the primary mode of relief in emergent situations, but the major breakthrough for this class of drug was

the advent of long-acting β2-agonists (LABA) such as salmeterol and formoterol, whose effects can last up to 12 hours at a time[106]. These agents are particularly important for asthma maintenance therapy, but are largely considered to be supplemental drugs. Overuse side effects of bronchodilators include tachycardia, hypertension, and tolerance to β-agonists.

Inhaled corticosteroids (ICS) have revolutionized management of asthma as soon as the disease came to be known as a chronic inflammatory disorder of the airways. The primary benefit of ICS use is that these drugs can quickly and efficiently dampen inflammation through effects on a wide range of targets. ICS diffuse across cell membranes, where they bind to cytoplasmic glucocorticoid receptors. This activates the receptors, which subsequently translocate to the nucleus and modulate the transcriptional activity of several target genes that have glucocorticoid response elements in their promoters[107]. ICS work primarily by suppressing the actions of pro-inflammatory transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP1) while simultaneously enhancing the activity of anti-inflammatory proteins such as inhibitor of nuclear factor-κB (IκBα), annexin-1, and IL-10[108]. However, whereas ICS are very effective at broadly suppressing airway inflammation, they do not alter the specific underlying mechanisms that contribute to asthma and thus cannot improve the natural course of disease. In addition, their use has been linked to a range of undesirable effects including candidiasis, dysphonia, adrenal insufficiency, bone loss, skin thinning, stunted growth, metabolic changes, and behavioral abnormalities despite their seemingly innocent safety profile[109].

Although ICS and bronchodilators have been solid agents of choice for asthma management, asthma-associated morbidity and economic burden are nevertheless increasing as disease prevalence continues to expand. Healthcare visits as well as the ability to purchase meds are vital to management, and cost barriers to obtaining these services often contribute to medical emergencies. In addition, 5-10% of asthmatics do not have well-controlled disease despite adherence to inhaled therapy (so-called "severe asthmatics"). These patients account for a disproportionate amount of healthcare spending on asthma, as

they are the most prone to hospitalization, high-volume medication consumption, and lost time from work. This has increased the need to find novel and improved therapies for asthma.

b. Targeted immunologic strategies for managing asthma

Despite the fact that our current pharmacologic agents for asthma are far from perfect, our treatment strategies for this disorder have evolved very little over the past century. A major contributor to this problem is that despite their shortcomings, existing therapies for asthma have many desirable qualities: they are relatively effective, safe and inexpensive. Moreover, the simple approach of pairing a LABA with an ICS for long-term asthma management has been supported by strong scientific rationale, as these two drugs have been shown to address complementary aspects of asthma pathophysiology[110]. Nevertheless, studies in small animal models of asthma have attempted to redirect our approach to asthma treatment, and many specific immunologic agents have been tried in humans. A handful of these treatments will be detailed within this section.

Mast cell stabilizers (e.g. sodium cromoglicate, nedocromil sodium) were first introduced as a treatment for asthma in 1968 and work by inhibiting chloride flux into mast cells, which increases their threshold for activation[107]. Newer mast cell inhibitors have been developed since this time, many of which inhibit mast cell chemotaxis and/or FcεRI-mediated mast cell activation. Other drugs such as montelukast and zileuton inhibit synthesis of mast cell- and eosinophil-derived leukotrienes, which enhance bronchoconstriction and eosinophil influx into airway tissues. However, the addition of leukotriene inhibitors to traditional ICS regimens has been shown to be less efficacious than traditional LABA supplementation for preventing asthma exacerbations and improving asthma symptoms, suggesting that mast cell inhibition is not sufficient for asthma management.[111]

Omalizumab (anti-hIgE IgG1k) was originally designed to reduce the sensitivity of asthmatic individuals to both inhaled and ingested allergens by binding free IgE and subsequently preventing cross-

linking of FcεRI receptors. It received Food and Drug Administration (FDA) approval in 2003 for treating patients with moderate to severe allergic asthma. Although omalizumab has been shown to reduce the number of asthma exacerbations, improve lung function, and increase quality of life for many of its users, major concerns have been raised over its high cost and long-term safety profile. In addition, omalizumab use is contraindicated in individuals who live in areas where parasite infection is endemic.

The critical role of Th2 cytokines in development of asthma has also led to the development of cytokine- and cytokine receptor-based immunotherapies for this disorder. This approach has required the use of blocking monoclonal antibodies, fusion proteins, and inhibitors of Th2-associated transcription factors such as GATA3 and STAT6[107]. IL-4 plays a critical role in the induction of Th2 cells and immunoglobulin isotype switching of B cells to IgE. Many studies have demonstrated that IL-4 blockade can attenuate allergic inflammation in animal models of AAD, however, phase 2 trials have not demonstrated efficacy of using humanized monoclonal Abs against IL-4 to attenuate disease.[112] Other studies have assessed the effects of using humanized, IL-5-specific monoclonal Abs to reduce asthma-associated eosinophilia. In a small, double-blind trial, these antibodies showed a dose-dependent reduction in local and systemic eosinophils that lasted for 3 months; however, this effect did not seem to attenuate AHR[113], thereby raising doubts about the efficacy of targeting single cell types and/or molecules for treatment of this complex disorder. The need for more effective immunologic strategies for managing asthma is clearly substantial.

c. Allergen-specific immunotherapy as an etiologic treatment for asthma

Allergen-specific immunotherapy (ASIT) is currently the only etiologic therapy for atopic disorders and has been in existence for over a century[114]. The primary difference between ASIT and other therapies (e.g. antihistamines, antileukotrienes, bronchodilators, ICS) for asthma is that ASIT actively restores long-term immunologic tolerance through repeated exposures to the triggering agent (i.e. is disease

modifying), whereas the rest merely provide a temporary and non-specific suppression of inflammation (i.e. are palliative). ASIT has been proven to be effective for treatment of allergy, allergic rhinitis, allergic conjunctivitis, and mild to moderate asthma. Its ability to lower clinical symptom scores has been shown to last 3 to 5 years post discontinuation of therapy[115-117]. In addition, administration of ASIT protects against development of new allergies; in one study examining children aged 5-8 years with HDM-induced asthma, half received ASIT for 3 years while the other half received symptomatic medications only[118]. Three years after discontinuation of ASIT, only 25% of children who received ASIT developed new skin test sensitivities, as compared to 67% of children in the control group. Finally, studies have indicated that when compared to traditional pharmacologic agents, ASIT reduces medical costs by up to 80% beginning as early as three months after initiation of therapy[119], suggesting that it has enormous economic incentives in addition to strong clinical advantages. ASIT is most commonly performed subcutaneously, but more recently has included sublingual application.

Mechanisms of ASIT

The ultimate goal of ASIT is to induce a state of peripheral immune tolerance to the triggering allergen. Although the specific mechanisms by which ASIT exerts its anti-inflammatory effects in asthmatic patients are actively being investigated, as of yet, its consequences remain incompletely defined due to the use of heterogeneous treatment protocols and treatment outcomes. Moreover, most ASIT studies have been conducted in humans and thus have been unable to evaluate the immunologic and physiologic changes occurring within the lungs. Nevertheless, several important patterns have emerged from studies investigating the clinical effects of ASIT. These studies have demonstrated that ASIT modifies the response of numerous cell types including DCs, T cells, B cells, and granulocytes. More specifically, several papers have noted that ASIT promotes the formation of inducible Treg populations, downregulates T effector (T_{eff}) responses, increases the ratio of IgG4 to IgE, and decreases the activity of basophils and mast cells. These specific mechanisms will be discussed in more detail within the following sections:

Tolerogenic effects on DCs and generation of Tregs: DCs in the submucosa of the airways play an important role in the immune response against newly encountered Ags, where, as mentioned previously, they can capture Ags and migrate to the T cell areas of the HLN. In the absence of pro-inflammatory signals (as does occur with ASIT), DCs maintain a partially mature phenotype[120] and display tolerogenic interactions with T cells in the lymph node[121], which skews T cells towards a regulatory (IL-10-secreting) phenotype. In addition, ASIT increases DC production of IL-10[120], which further downregulates expression of costimulatory molecules on DCs and promotes subsequent T cell anergy.

The primary mechanism by which ASIT is thought to benefit patients with asthma and allergy is through DC-mediated generation of iTregs. Long-term ASIT in a murine model of OVA-induced asthma was associated with an increased frequency of $Foxp3^+$ Tregs in the HLN that correlated with a reduction in allergic airway inflammation and AHR[122]. Furthermore, depleting these Tregs during ASIT inhibits the suppression of disease associated with this therapy[123]. Human studies have robustly supported the conclusion that ASIT stimulates specific increases in Treg numbers[124,125], expression of Foxp3[51], and functional activity[51]. Other human studies have shown no differences in frequency of $Foxp3^+$ Tregs in the blood of asthmatic patients receiving HDM-ASIT, but have shown specific increases in levels of IL-10-producing $Foxp3^+$ T cells in the blood, a difference that became even more striking when cells were subsequently cultured *in vitro* with HDM[126]. Furthermore, ASIT has been shown to restore the suppressive activity of $Foxp3^+$ Tregs[51]. This suppressive activity may be related to the ability of Tregs to produce anti-inflammatory cytokines (IL-10, TGF-β) and/or expression of CTLA-4, which can curb the responses of T_{eff} cells and skew immunoglobulin production towards protective IgG4 (through IL-10) and IgA (through TGF-β) subtypes[67] (see below for more details). Skewing this balance between allergen-specific Treg and T_{eff} cells appears to be critical for the correction of allergic immune responses against innocuous substances[127].

In addition to Foxp3$^+$ Tregs, several studies have demonstrated that ASIT increases the number of IL-10-secreting CD4$^+$ T cells[128] and Tr1 cells[122,127], both of which have been thought to play a major role in the efficacy of ASIT. Specifically, increased local Tr1 cells have been associated with elevated IgG4 levels and blocking activity due to their enhanced production of IL-10. In a murine study of OVA-ASIT, neutralization of IL-10 signaling abrogated the immunosuppresive effects of ASIT and led to increased AHR, eosinophilia, IgE titers, and evidence of airway remodeling[122].

Moreover, some studies have shown that ASIT also increases IL-10 production by non-dendritic cell APCs, including monocytes and tissue macrophages[129,130]. These cells may, in turn, help skew T cells towards a regulatory Tr1 phenotype.

Downregulation of T effector cells: T cells of asthmatic patients typically undergo extensive proliferation when they come in contact with their cognate allergen. Early studies demonstrated that this proliferative response was suppressed in patients receiving ASIT[131]. A series of elegant studies later demonstrated that T cells undergo a state of peripheral anergy following ASIT in patients with bee venom allergy, as marked by decreased production of Th2 cytokines to levels observed in nonallergic individuals[132]. In most cases, the levels of Th2 cytokines produced were shown to inversely correlate with duration of ASIT. These observations clearly indicated that ASIT accomplishes its clinical effects, in part, through suppression of Th2 effector responses. Additionally, allergen-specific T effector (T_{eff}) cells from patients receiving ASIT were shown to produce increased levels of IL-10[129], and neutralization of IL-10 was specifically shown to break T cell anergy and restore the ability of T cells to proliferate and produce Th2 cytokines. Furthermore, ASIT has been associated with a shift in CD4$^+$ T cells from a Th2 phenotype to an INFγ-secreting Th1 phenotype[133,134]. This change in cytokine expression may assist in mediating the clinical benefits of ASIT by regulating pathogenic Th2 responses. ASIT also alters responses of allergen-specific memory T- and B-cells[115], thus explaining the persistence of its effects long after discontinuation of treatment.

Effects on immunoglobulin responses and B cells: Upon initial response to an allergen, low affinity IgG1 antibodies are generated against the allergen. In healthy individuals that do not develop allergy, repeated exposure to the allergen generates high affinity IgG4 antibodies against the allergen, whereas in atopic individuals, allergen challenge results in production of IgE[129]. Many of the benefits of ASIT appear to be mediated through alterations in allergen-specific immunoglobulin profiles, specifically, decreases in allergen-specific IgE/IgG4 ratios[127] through enhanced Treg activity. The induction of IgG4 in B cells by Tregs has been shown to be dependent on GITR and IL-10 and can be enhanced by TGF-β[135] (IL-10 consequently also inhibits IL-4-mediated class switching to IgE). It is thought that generation of Ag-specific IgG antibodies directed against the same epitopes as Ag-specific IgE may directly compete for allergen binding and exert a "blocking" effect on IgE[121]. In support of this theory, removal of IgG4 activity in the sera of patients who received ASIT resulted in a nearly complete loss of the inhibition of allergen-IgE binding to FcεRII on B cells[130]. However, antibody responses that are induced with ASIT have been shown to be functionally heterogeneous, and thus the specific mechanisms by which IgG may benefit ASIT patients is not known (Note: Induction of IgG antibodies specific for epitopes not bound by allergen-specific IgE would not exert a "blocking" effect and may even amplify cross-linking of allergen-bound IgE-FcεRI complexes). A second possibility is that induction of IgG decreases the number of B cells that undergo class switching to IgE.

In addition to IgE, studies have also shown that increased levels of allergen-specific IgA (induced by TGF-β) have been associated with successful ASIT[136]. Both IgA and IgG4 represent non-inflammatory isotypes that can counter the pro-inflammatory effects of IgE.

Suppression of mast cells/basophils: Starting from the first injection, ASIT modifies the susceptibility of mast cells and basophils to degranulation. Studies have shown that venom immunotherapy decreases IgE-mediated histamine and leukotriene release[137]. This decrease in mast cell and basophil activity has been shown to occur much earlier than the decrease in allergen-specific IgE and

skin test reactivity in HDM-sensitive asthmatic subjects[138]. Both IL-10 and INFγ release by T cells have been implicated in this process[137].

Adverse effects of ASIT

Variability in safety and efficacy profiles of ASIT regimens has limited its widespread application within the clinic. One of the major hurdles in the popularization of subcutaneous ASIT is the potential for side effects, which range from mild symptoms to life-threatening anaphylaxic reactions. Given this concern, practice guidelines currently recommend that patients receive ASIT in a supervised medical facility, where they should be monitored for thirty minutes post injection. Unfortunately, this inconvenience has decreased the utility of ASIT for many patients that are unwilling to comply with these procedural recommendations. Thus, alternative methods for application with improved safety profiles are being actively investigated. Sublingual ASIT carries a reduced risk of anaphylaxis when compared to subcutaneous ASIT and can thus be administered in the comfort of one's home; however, the efficacy of sublingual ASIT is lower than in subcutaneous ASIT[121]. Epicutaneous ASIT is also currently being explored. Additionally, ASIT regimens that utilize recombinant T and B cell epitopes (as opposed to the entire, intact allergen) are currently being explored for their potential to decrease severe side effects

IV. Mouse models of asthma: what have they shown us?

a. Allergic airway disease

Animal models are valuable tools for exploring the processes that contribute to the pathogenesis of human disease. In addition to the obvious advantages of safety and cost effectiveness relative to studying disease in patients, animal models are far less medically complicated, making it easier to isolate the disease process(es) of interest. Most importantly, animal models allow us to dissect (often literally) the mechanisms that contribute to the diseases we study through use of invasive, and potentially lethal,

procedures. It would, for example, be difficult to understand the processes that contribute to the development of asthma if one could not isolate cells from the lung compartments. As a society, we have invested billions of dollars into advancing the molecular tools and techniques that are currently available to answer our most pressing scientific questions. The advent of experimental asthma models in combination with the ability to selectively inactivate genes has allowed us to explore a variety of avenues that may contribute to asthma pathogenesis. Although the translatability of our findings are always in doubt whenever we utilize animal models, the benefits of this approach undoubtedly outweigh the disadvantages: animal models stand firmly as the translational bridge between the boundaries of *in vitro* work and large-scale clinical trials.

We have gained a vast array of knowledge about the mechanisms that contribute to the development and prevention of asthma using mouse models of *allergic airway disease (AAD)*, the murine "equivalent" of asthma. Although mice with AAD do not manifest with all of the clinical signs and symptoms of human asthma (e.g. wheezing, dyspnea, cough), they recapitulate many immunological and pulmonary features associated with the disease, including eosinophilic infiltration in the lungs, activation of Th2 lymphocytes, and evidence of airway obstruction through mucus overproduction and AHR. Thus, much can be learned about human asthma from models of AAD.

Note: The majority of murine studies that focus on AAD are conducted in either C57BL/6 or BALB/c mice. While each strain has distinct advantages, it is important to note that the features of AAD may manifest slightly differently depending on which strain is used, with BALB/c mice traditionally demonstrating more significant AHR responses. The Thrall laboratory conducts its studies exclusively in C57BL/6J mice, a model which has proven to yield high quality, reproducible results. However, all major findings from the Thrall laboratory, including development of local inhalational tolerance (*discussed in Section IVd*) have also been confirmed in BALB/c mice.

b. Ovalbumin (OVA)-induced AAD model

The majority of experimental work on asthma has been conducted using the model Ag OVA, as it is easy to work with and yields reproducible results. It has been so extensively studied in both acute and chronic asthma models that it forms the basis for comparison for all future studies involving different model Ags. Moreover, technologies to specifically track OVA-specific cells and proteins (e.g. OVA TCR-transgenic mice, SIINFEKL [OVA dominant epitope] tetramers, anti-OVA immunoglobulins) are abundant. Nevertheless, concerns about the OVA model have begun to arise despite the fact that it has been so useful in elucidating the fundamental immune processes involved in the development and resolution of asthma. Although OVA is clearly capable of eliciting AAD in mice, it is not a significant cause of human asthma and lacks many of the complex immunological characteristics of the more clinically relevant allergens. In addition, the route of sensitization commonly utilized with this model (intraperitoneal [i.p.] sensitization) does not relate to the natural (inhaled) route by which humans become sensitized. Furthermore, animals will only develop AAD if they are first immunized against OVA using aluminum hydroxide (alum), an artificial protocol that biases the immune system towards a Th2-driven response prior to OVA aerosol exposure[139]. Thus, although previous investigations in long-term models of OVA-induced AAD have added tremendously to our knowledge of the tolerogenic mechanisms utilized by the mucosal immune system to suppress allergic inflammation, OVA is far less structurally and immunologically complex than the majority of human allergens[140]. Therefore, despite their tremendous utility, OVA-induced models of AAD are not likely to involve the entire scope of inflammatory processes that are involved in asthmatic responses. The need persists for more physiologically relevant models of human asthma (see section IVd).

c. House dust mite (HDM)-induced AAD model

Composition of HDM

Dust mites are microscopic, non-parasitic arthropods that generate potent allergic reactions in susceptible individuals. It has been estimated that 50-85% of all asthmatics harbor an HDM allergy[141], thus making HDM the most common allergen worldwide. Over 50,000 species of HDM have been identified, but the two most common causes of allergy in the United States are *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). Unlike OVA, HDM is an extremely complex Ag that triggers widespread activation of both innate and adaptive immune systems. The allergic potential of HDM rests within its body fragments as well as within its fecal pellets. These pellets are typically 10-40 μm in diameter and can thus be inhaled and deposited within the airways with ease.

What makes HDM such a popular culprit in allergy is its ubiquitous nature; mites feed primarily on dead human skin cells, the major component of dust. Thus, HDM can be found in any location where dust is present. Mites typically settle on surfaces such as bedding, pillows, and upholstered furniture, which serve as their nests*. Eliminating dust, decreasing humidity, and washing clothing and sheets in hot water are the only methods known to efficiently decrease HDM concentration.

Protease activity is a common feature of most human allergens, including fungi, pollen, pet dander, and HDM. Mites and their fecal pellets contain over 23 different groups of proteolytic enzymes (named by species and group number) that are vital for initiation of an allergic response. Group 1 allergens (e.g. Der f 1, Der p 1) are cysteine proteases that share sequence identity with the catalytic site of papain, while group 3, 6, and 9 allergens are serine proteases that account for 79% of the proteolytic activity of HDM[141]. These proteolytic enzymes facilitate cleavage of epithelial tight junctions in the airway, allowing allergen delivery from the airway lumen to submucosal APCs following long-term exposure. In addition to their effects on junctional proteins, these proteases can react with cell surface protease activated receptors (PARs) in the airways, resulting in cytokine/chemokine production from

* Unlike most human allergens, HDM does not typically remain airborne.

epithelial cells, enhanced degranulation of mast cells and eosinophils, bronchial smooth muscle contraction, and maturation/proliferation of collagen-producing fibroblasts.

As mentioned in Section IIb, an important stage in asthma development is the sensitization phase, which requires the presence of an adjuvant. LPS is a potent adjuvant that is found in abundance in HDM extract. Thus, a major advantage of using inhaled HDM rather than aerosolized OVA to induce AAD is that disease results after mucosal sensitization in the lungs (as is presumed to occur in humans) and does not require peripheral sensitization with an external adjuvant. However, it is noteworthy to mention that the role of LPS in the initiation of asthmatic responses is indeed complex, and timing and pattern may have important effects on the development of atopy[141].

In addition to its LPS activity, HDM-associated group 2 allergens (Der p 2, Der f 2) belong to the MD-2-related lipid-recognition domain family and facilitate aggregation of TLR4. Thus, these proteins help promote LPS-induced TLR4 activation in the absence of its co-receptor MD2. This is of tremendous importance because bronchial epithelial cells express TLR4 but very little MD-2[142], so group 2 allergens are essential for the airway epithelia to sense endotoxin in HDM extract.

LPS induces innate immune responses through both TLR4- and TLR2-signaling activity. While TLR2 appears to be dispensable for generating a robust allergic response to HDM, $TLR4^{-/-}$ mice have been shown to demonstrate significant attenuation of eosinophilia, Th2 cytokine production, and AHR, suggesting that HDM-associated inflammation is, in part, TLR4-dependent[143]. Furthermore, upregulation of TLR4 has been shown to increase on bronchial epithelial cells following airway challenge with HDM[144], and its expression on structural cells is both necessary and sufficient for DC activation and T cell priming in response to HDM[8]. Stimulation of the TLR4 pathway leads to recruitment of cytosolic adaptor proteins (e.g. MyD88, Trif, Tram), which subsequently activate protein kinases that upregulate production of various transcription factors involved in pro-inflammatory gene expression. These include GM-CSF (involved in alveolar macrophage maturation) and co-stimulatory molecules on DCs and macrophages[144] in addition to TSLP, IL-25, and IL-33.

In addition to a variety of immunogenic epitopes, HDM extract also contains a variety of additional components, including chitins and β-glucans. The β-glucans (a component of fungal and bacterial cell walls) bind to dectin molecules expressed on the surface of myeloid cells. This ligation has been shown to result in the secretion of CCL20, a chemokine which stimulates recruitment of immature DCs[12]. Chitin is a major component of the mite exoskeleton and serves as a multifaceted adjuvant that stimulates accumulation of eosinophils and basophils[145] as well as activation of macrophages[141]. Asthmatic individuals have been shown to have elevated levels of chitinases when compared to healthy individuals, which is important because these molecules may play a direct role in the augmentation of Th2 responses[146].

The tremendous complexity of HDM, the most globally recognized human allergen, demonstrates why OVA is no longer the most effective tool for understanding the complex immune reactions that occur in asthma. Studying responses to inhaled HDM (particularly long-term HDM exposure) will be much more promising for downstream translation. However, it will certainly be valuable to compare any findings from these HDM studies to those from long-term OVA models, as publications involving long-term HDM exposure have been quite limited. Moreover, comparing and contrasting responses to these two different allergens, one of which is quite complex and the other of which is fairly simple, may have tremendous power in teasing apart various mechanisms of asthma pathogenesis and disease resolution.

d. Chronic OVA exposure leads to development of local inhalational tolerance (LIT): a summary of the Thrall laboratory findings to date

Almost 20 years ago, Dr. Roger Thrall set out on a mission to study lung remodeling in the setting of asthma. To create a model of chronic asthma, his laboratory immunized C57BL/6 mice with three weekly intraperitoneal injections of OVA and aluminum hydroxide, followed by up to six weeks of OVA aerosolization. While short-term exposure (3 to 10 days) to OVA aerosol resulted in hallmarks of AAD

(e.g. local leukocytosis, bronchoalveolar lavage (BAL) eosinophilia, peribronchiolar/perivascular lung inflammation, and AHR), the laboratory was surprised to find that long-term (6 weeks) OVA exposure led to resolution of AAD rather than chronic remodeling. This resolution phase was marked by drastic decreases in BAL leukocytes, eosinophils, Th2 cytokines, perivascular/peribronchiolar lung inflammation, and AHR[147] and was presumed to represent a state of immunologic tolerance to OVA. Follow-up studies demonstrated that this tolerance was dependent upon continuous Ag exposure[148]. In addition, it was found that this tolerance was localized to the lung, as systemic responses to IgE were still intact upon footpad challenge[149]. In turn, resolution of AAD with long-term, continuous OVA exposure was termed 'local inhalational tolerance' (LIT).

Over the past decade, the Thrall laboratory has been actively investigating the mechanisms that contribute to the development of LIT with long-term Ag exposure. Our group believes that understanding these mechanisms may help to identify immunologic defects that could predispose certain individuals to atopy and in turn, could lead to more targeted approaches for treating asthma. Previous findings from the laboratory have demonstrated that OVA-induced LIT is associated with immunoregulatory shifts in the leukocyte composition within the lungs; namely, a significant accumulation of Tregs within the HLN[150]. Adoptive transfer of cells from the HLN of LIT mice into $RAG^{-/-}$ mice led to significant decreases in eosinophil numbers and lung inflammation after OVA challenge when compared with mice that received HLN cells from AAD mice. This suggests a causal role for HLN Tregs in the development of LIT. Similar findings were noted when LIT HLN B cells were adoptively transferred into OVA-sensitized mice prior to onset of aerosolization[151]. Follow-up studies supported this finding by demonstrating that adoptive transfer of LIT-induced $CD5^{+}$ B regulatory cells (Bregs) from the HLN led to induction of Tregs within the lungs in conjunction with suppression of AAD[152]. Thus, the OVA-LIT model has effectively shown that tolerance develops with long-term exposure to inhaled Ag and that Tregs and Bregs are implicated in this process. However, tolerance to more complex, human-relevant Ags (e.g. HDM) has never before been demonstrated. Thus, although it is speculated that non-asthmatic individuals can

develop immune tolerance to HDM, this has never before been experimentally demonstrated with long-term HDM exposure (*see Chapter 3 for more details*).

V. Hypothesis and project summary

Murine studies have clearly shown that long-term, continuous exposure to the model Ag OVA results in development of immune tolerance and resolution of AAD. These studies have played a vital role in understanding the mechanisms that contribute to the normal development of immune tolerance against allergens, a process that is perhaps mimicked in the 90% of people who do not have symptoms of asthma. However, as previously stated, OVA is far less complex than the majority of human allergens and is not a clinically-relevant cause of asthma. On the other hand, HDM is a widespread trigger of human allergy and affects anywhere from half to three quarters of all asthmatic individuals worldwide. Thus, one might postulate that to inflict such a large proportion of the population, the immune system cannot readily develop tolerance to HDM in the same manner that it does with OVA. Furthermore, prior studies in the literature have not previously demonstrated resolution of AAD with long-term HDM exposure, though this question has not been definitively addressed by any other group.

The goal of my studies was to determine whether immune tolerance can be generated following long-term exposure to HDM, the most ubiquitous and important allergen in the world. As previously stated, this question has gone unanswered by other researchers that have conducted studies in long-term models of HDM-induced AAD. Understanding if (and how) tolerance develops to HDM would be critically important for 1) defining the mechanisms by which HDM results in asthma in susceptible individuals and 2) aiding in the development of enhanced or novel treatments for HDM-induced asthma and allergy.

Given the immunologic complexity of HDM and the high prevalence of HDM-induced asthma and allergy, I hypothesized that immune tolerance (and subsequent resolution of AAD) would not be readily mounted with long-term HDM administration as it is with OVA. Results from this dissertation project

have demonstrated that while short-term and intermediate-term intranasal HDM exposure (2-5 weeks) result in cardinal features of AAD, long-term HDM instillation (11 weeks) does, in fact, stimulate immunologic tolerance to the traditional, AAD-associated Th2 response. However, unlike with OVA, tolerance to HDM was associated with *suppression* of disease but not resolution of lung inflammation (Chapter 3). Nevertheless, this state of suppression was marked by an increase in both $Foxp3^+$ Tregs and $IL\text{-}10^+$ AMs at the site of inflammation, the latter of which is unique to the HDM model and has never before been observed with long-term OVA exposure. Although these AMs were found to be nonessential for immune tolerance against HDM (Chapter 4), *in vitro* studies demonstrated that AMs from tolerant mice were indeed capable of inducing $Foxp3^+$ Tregs and may thus have had a *contributory* role in tolerance formation within the context of our continuous, long-term HDM model. Interestingly, parallel studies in a clinically relevant, murine model of ASIT (Chapter 5) did not support a critical role for these AMs in tolerance induction.

Figures

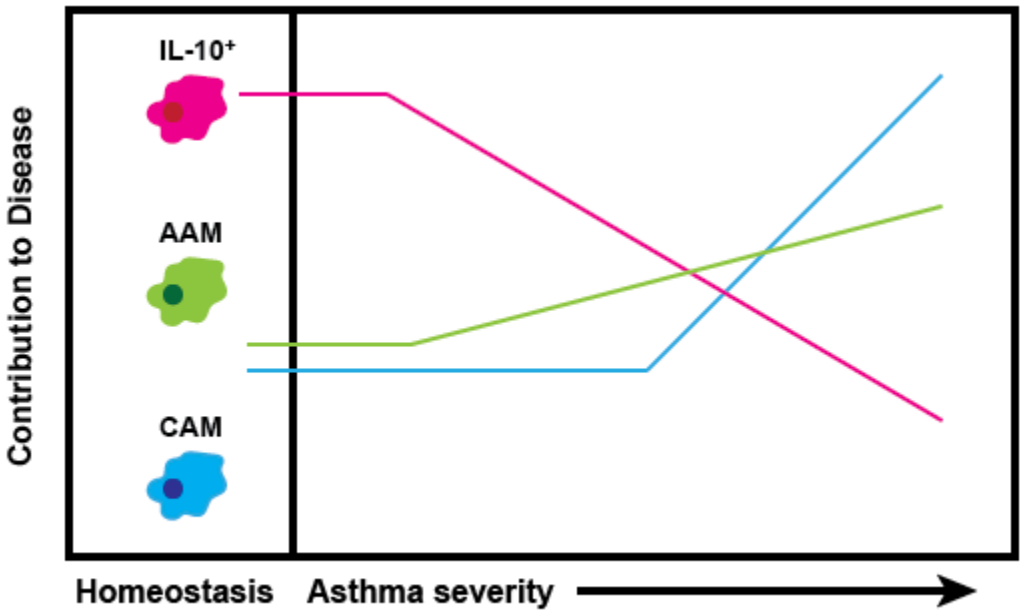

Figure 1. The contributions of three major macrophage subtypes to the severity of allergic asthma in mice and humans

CHAPTER 2

MATERIALS AND METHODS

Animals

Female C57BL/6J (wild type; WT) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10$^{-/-}$) mice, 6-8 wks of age, were purchased from Jackson Laboratory (Bar Harbor, ME) and conventionally housed in plastic cages with corncob bedding. B6(Cg)-*Il10*$^{tm1.1Karp}$/J ("Vert-x"; IL-10^{gfp}) mice (originally obtained from Christopher Karp and generously provided by Leo Lefrançois) were bred in the Center for Comparative Medicine at the University of Connecticut Health Center, and 6-8 wk old mice of both male and female sexes were used. Data was combined for both sexes because no differences were observed in disease phenotype between male and female Vert-x mice.

The animal room was maintained at 22-24°C with a daily, 12 hour light/dark cycle. Chow and water were supplied *ad libitum*. The protocols for animal use were approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center (protocol #100331-1114).

House Dust Mite Exposure Protocol

Mice were lightly anesthetized with vaporous isoflurane and intranasally (*i.n.*) instilled with droplets containing 25 μg lyophilized HDM extract (equal mixture of *D. pteronyssinus* and *D. farinae*, Greer Laboratories, Lenoir, NC) solubilized in 50 μl phosphate buffered saline (PBS). This dose has previously been associated with peak inflammation in response to i.n. HDM and has been commonly utilized to

induce AAD[153,154]. *D. pteronyssinus* and *D. farinae* were selected as model Ags based on the fact that they comprise the two most common perennial indoor HDM species[141]. Total lipopolysaccharide (LPS) content in the HDM extract was 1250 endotoxin units per mg HDM (equal to approximately 0.1 μg LPS/mg HDM[155]; 0.01% contamination).

HDM was administered for five consecutive days, followed by two days of rest, for up to 11 consecutive wks (*Fig 2; henceforth referred to as "continuous" exposure model)*. Control groups received equal volumes of i.n. PBS in a time-matched manner. Mice were sacrificed at after either short-term (2 wk), intermediate-term (5 wk) or long-term (11 wk) Ag exposures. All mice were sacrificed 72 hours post final Ag challenge.

In select experiments, two groups of animals were discontinuously exposed to HDM extract for 11 wks (*Fig 3*). Group 1 mice ($DiscR^-$) were administered HDM for 5 wks and then discontinued from HDM exposure for the following 6 wks without any subsequent rechallenge. Group 2 mice ($DiscR^+$) were administered HDM for 5 wks, discontinued from HDM exposure for the following 4 wks, and then rechallenged with 2 wks of HDM Ag.

BAL, Blood, and Tissue Analysis

At sacrifice, BAL fluid, hilar lymph node (HLN), and lung tissue from each animal were harvested and processed for isolation and enumeration of leukocytes. For isolation of BAL leukocytes, lungs were lavaged *in situ* with five, 1-ml aliquots of saline and pelleted at 600 x g for 10 min at 4^0C. HLNs were mechanically disrupted into single-cell suspensions and filtered through a 100 μm Nitex screen (ELKO Filtering Co, Miami, FL). Lungs were digested with 150 U/ml collagenase (Life Technologies, Grand Island, NY) and mechanically disrupted into single-cell suspensions, after which erythrocytes were lysed via Tris-buffered ammonium chloride (TAC) solution (9 parts 0.83% w/v NH_4Cl; 1 part 2.57% w/v Tris; pH 7.0) at room temperature. For isolation of blood leukocytes, 100 μl of blood was drawn from the right

ventricle and passed through heparinized capillary tubes (Fisher Scientific, Hanover Park, IL) to prevent clotting. Red blood cells were lysed with TAC solution at 37^0C.

For all tissue samples, total nucleated cell counts were obtained using a hemocytometer with nigrosin dye exclusion as a measure of viability. Cytospin preparations of BAL fluid were stained with May-Grünwald and Giemsa for differential cell analysis via light microscopy. Multinucleated macrophage frequency was determined by manual counts after staining.

Flow Cytometry

Cells isolated from the BAL, HLN, and lung tissue were analyzed via flow cytometry using the following monoclonal antibodies: anti-Siglec-F (E50-2440; BD Biosciences), anti-F4/80 (BM8.1; Tonbo Bioscience), anti-CD11b (M1/70; eBioscience), anti-CD11c (N418; eBioscience), anti-MHC Class II I-A/I-E (M5/114.15.2; Tonbo Bioscience), anti-CD3 (145-2C11; Tonbo Bioscience), anti-CD4 (RM4-5; Tonbo Bioscience), anti-PD-1 (RMP1-30; Biolegend), anti-CD25 (PC61.5, Tonbo Biosciences), and anti ST2 (RMST2-2; eBioscience). Samples were stained as previously described[152]. Briefly, cells were washed in PBS containing 0.2% bovine serum albumin and 0.1% NaN_3. Aliquots containing 10^5-10^6 cells were incubated with anti-mouse CD16/CD32 (FcBlock; eBioscience) for 15 min. Cells were then stained with 100 µl of appropriately diluted Live/Dead Fixable Blue Dead Cell Stain (Invitrogen) and surface antibodies for 30 min at 4°C. Following staining, cells were fixed with 4% paraformaldehyde. For identification of Tregs, cells stained with anti-CD3 and anti-CD4 were treated with Foxp3/Transcription Factor Fixation/Permeabilization buffer (eBioscience) according to the manufacturer's instructions and stained with anti-Foxp3 (FJK-16s; eBioscience). Samples were run with corresponding isotype controls on a BD LSR II (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo (Tree Star Software, Ashland, OR).

Confocal microscopy

Lung tissue was fixed in PLP buffer overnight at 4º C, washed with P-buffer, and placed in 30% sucrose for 6 hours at 4º C prior to cryopreservation in OCT. 20 μm thick sections were blocked and stained in 1X PBS supplemented with 0.05% Tween-20, 0.3% Triton-X 100, 2% FBS, 2 % goat serum, and 0.5% Fc block. Lung sections were stained for epithelial cells, AMs, and CD4 T cells using anti-CD326 (G8.8; eBioscience), anti-F4/80-APC (BM8; Biolegend), anti-CD11c-PE (N418; Biolegend), and anti-CD4-BV421 (GK1.5; BD Biosciences). Sections were imaged on an LSM 780 microscope (Zeiss, North Chesterfield, VA) and confocal images were analyzed using the Imaris (Bitplane AG, Zurich, Switzerland) software program.

Histology

After sacrifice, unmanipulated lungs from animals not subjected to BAL were removed, fixed with 4% buffered formalin, and processed in a standard manner. Tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) for mucus production at the University of Connecticut Health Center Research Histology Core. Sections from all five lobes were examined via light microscopy in their entirety and images were taken from representative regions of inflammation. Pathological scoring for inflammation and mucus production was performed in a blinded manner by five reviewers on a severity scale of 0 to 3 as similarly described[156]. For inflammation scores, a value of 0 was assigned when no inflammation was detectable, a value of 1 for mild peribronchiolar/perivascular cuffing with inflammatory cells, a value of 2 for significant peribronchiolar/perivascular clustering, and a value of 3 for significant clustering and airway remodeling. For mucus scores, a value of 0 was assigned when no mucus was present, a value of 1 for occasional and punctate mucus staining in the airways, a value of 2 for presence of mucus rings in less than 10% of airways, and a value of 3 for presence of mucus rings in greater than 10% of airways.

Determination of Immunoglobulin Levels

At sacrifice, blood was collected from mice via cardiac (right ventricular) puncture, allowed to clot, and serum was isolated.

For detection of HDM-specific IgE in serum, 96-well Nunc MaxiSorp flat bottom plates (Thermo Scientific) were coated with 10 ug/ml HDM extract in sodium bicarbonate buffer (pH 9.5) for 16 hours at 4^0C. Plates were washed with 0.05% PBS/Tween 20 and blocked for 1 hour at 37^0C with BD OptEIA Assay Diluent (BD Biosciences, San Diego, CA). After washing, serum samples were incubated at room temperature for 1.5 hours and two-fold serial dilutions were performed (range: 1/20 to 1/2560). Plates were washed eight times, whereafter samples were labeled with Biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech) followed by Streptavidin-HRP (BD Biosciences).

Detection of HDM-specific IgG1 was performed as similarly described[153]. Plates were coated with 2 ug/ml HDM extract in sodium bicarbonate buffer for 16 hours at 4^0C. Plates were washed and blocked for 1 hour at room temperature with BD OptEIA Assay Diluent (BD Biosciences, San Diego, CA). After washing, serum samples were incubated at room temperature for 1.5 hours and ten-fold serial dilutions were performed (range: 1/20 to 1/200,000,000). Plates were washed eight times, whereafter samples were labeled with Biotin-SP-conjugated goat anti-mouse IgG Fcγ Subclass 1 specific antibody (Jackson ImmunoResearch, West Grove, PA) followed by Streptavidin-HRP (BD Biosciences).

Detection of total IgE was performed as similarly described[157]. Plates were coated with 2 μg/ml rat anti-mouse IgE (R 35-72) diluted in sodium bicarbonate buffer overnight at 4°C. Subsequently, coated wells were blocked with 1X NAP-BLOCKER (G-Biosciences, St. Louis, MO) for 1 hour at room temperature. After washing, serum samples were incubated for 1 hour at room temperature, washed, and developed with Goat anti-mouse IgE-HRP (A1-H12) for 30 min at room temperature.

For all assays, labeling was followed by incubation with 3,3',5,5'-Tetramethylbenzidine (TMB), and the reaction was stopped with an equal volume of 1 M phosphoric acid. Dual absorbance at 450 nm and 570 nm was measured with a Bio-Rad (Hercules, CA) model 480 microplate reader. For total IgE, a standard curve was created using mouse IgE kappa anti-TNP (C38-2).

Quantification of Cytokines

Detection of cytokines was performed using a custom Milliplex MAP Mouse Cytokine Magnetic Bead Panel (EMD Millipore, Billerica, MA). Serum samples were diluted two-fold and BAL supernatant samples were concentrated ten-fold using Amicon Ultra Centrifugal Filter Units, 10K MWCO (Millipore). Cells from lung tissue were processed into single cell suspension and lysed via sonication. Cells were spun at 300xg for 5 min at 4^0C and supernatant was concentrated ten-fold fold as described above. Total protein levels were quantified via BCA assay; equal amounts of protein were run from each sample. All samples were run in duplicate as recommended by the manufacturer.

Measurement of Airway Hyper-Reactivity

Airway reactivity was assessed on the basis of total respiratory system resistance (Rrs) response to increasing doses (0-100 mg/ml) of acetyl-β-methacholine chloride (Sigma-Aldrich, St. Louis, MO). Mice were anesthetized i.p. with 100 μg/kg nembutol sodium solution (Ovation Pharmaceuticals Inc, Deerfield, IL). Mice underwent tracheostomies and were mechanically ventilated using the flexiVent System (SCIREQ, Montreal, CA). To paralyze respiratory muscles prior to onset of methacholine exposure, animals were administered 500 ng/kg i.p. pancuronium bromide (Sigma-Aldrich, St. Louis, MO). Airway reactivity was determined by assessing forced oscillatory mechanics every 10 seconds for 4 minutes following each methacholine challenge. EKG measurements were used to ensure viability for the duration of methacholine challenge.

Treg induction assay

Freshly isolated $CD4^+CD25^-$ Teff cells were obtained from the spleens of naïve Foxp3<tm1Kuch> ($Foxp3^{gfp}$) mice (originally obtained from Vijay Kuchroo and generously provided by Robert B. Clark) using magnetic bead isolation (Miltenyi Biotech, Aurburn, CA) with >98% purity. These Teff cells were cocultured with viable AMs (Siglec-$F^+CD11c^+F4/80^+$) sorted from the lungs of short-term or long-term

HDM exposed mice or long-term PBS exposed mice at a 1:1 ratio (0.15 x 10^6 Teffs, 0.15 x 10^6 AMs) in the presence of soluble αCD3 (0.5 μg/ml; BD Biosciences) ± recombinant TGF-β (1 ng/ml; R&D Systems, Minneapolis, MN). The cultures were performed in RPMI 1640 supplemented with 10% FCS, 200 μg/ml penicillin/streptomycin, 50 μM 2-mercaptoethanol and 4 mM L-Glutamine in 96-well round-bottom plates. After 72 hours, cells were taken out from the wells, and the intracellular expression of Foxp3 was assessed on gated $CD4^+$ T cells via GFP expression.

In select experiments, viable lung AMs were cocultured with naïve T cells in the presence of soluble αCD3 and recombinant TGF-β ± anti-IL-10 neutralizing antibody (0.5 μg/ml; JES5-2A5; BD Biosciences) and anti-IL-10R neutralizing antibody (0.5 μg/ml; 1B1.3a; BD Biosciences) as described above. After 72 hours, cells were taken out from the wells, and the intracellular expression of Foxp3 was assessed on gated $CD4^+$ T cells via GFP expression.

Clodronate liposome administration

Clodronate or PBS control liposomes were purchased from FormuMax (Palo Alto, CA) and administered i.n. once per week (7 mg/ml; 50 μl per injection) from weeks 5-9 of continuous HDM challenge to deplete alveolar macrophages (*Fig 4*). Control liposomes were administered at equal volumes in a time matched manner. Animals were sacrificed 11 weeks post initial HDM challenge.

Allergen-specific immunotherapy (ASIT) administration

Subcutaneous ASIT was administered in the nape of the neck for either short-term or long-term periods. Short-term ASIT was performed as follows: mice were continuously administered i.n. HDM as shown in Figure 2 for a period of 5 wks. Biweekly, subcutaneous ASIT with a standardized HDM extract (Allergy Laboratories, Oklahoma City, OK),was initiated following development of early AAD (2 wks) and continued for the remaining three wks (total of 6 injections) at either high (200 allergen units [AU]) or low (20 AU) doses. Control animals received equal volumes (200 μl) of vehicle (normal saline phenol) in a time-matched manner. Mice were sacrificed 72 hours post final HDM instillation (*Fig 5A*).

Long-term ASIT was performed as follows: mice were administered i.n. HDM in a DiscR^{+} manner over an 11-wk period as shown in Figure 3A. Biweekly, subcutaneous ASIT with a standardized HDM extract (Allergy Laboratories) was initiated following development of early AAD (2 wks) and continued for the remaining nine wks (total of 18 injections) at either high (200 AU) or low (20 AU) doses. Control animals received equal volumes (200 μl) of vehicle (normal saline phenol) in a time-matched manner. Mice were sacrificed 72 hours post final HDM instillation (*Fig 5B*).

Statistical Analysis

Statistical comparisons between groups were made via one-way analysis of variance (ANOVA) followed by a Neuman-Keuls *post hoc* test. Airway hyper-reactivity and serum immunoglobulin curves were compared via area under the curve (AUC) measurements. One-way ANOVA was then performed on AUC data with a Neuman-Keuls *post hoc* test. Cytokine data and histopathology scores were compared via Kruskal-Wallis test with Dunn's Multiple Comparison *post hoc* test. For all data, values of $p<0.05$ were used as the significance threshold. All statistical analysis was performed using the GraphPad Prism (La Jolla, CA) statistical software package.

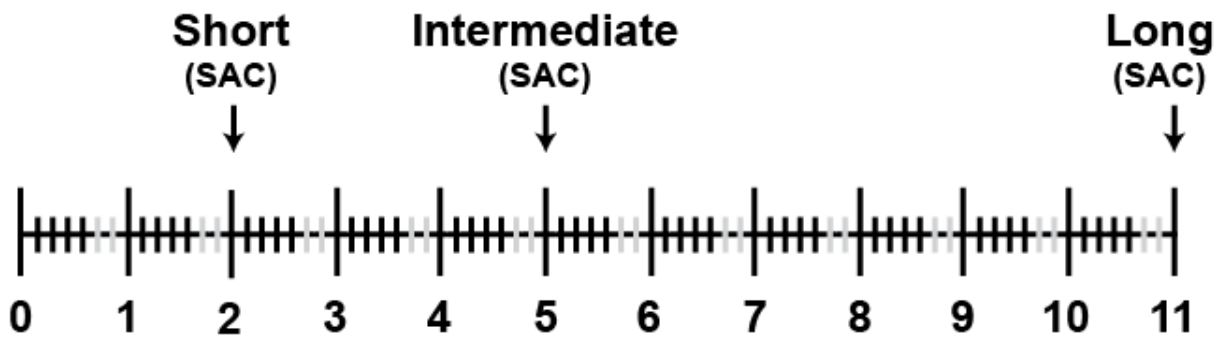

Figure 2. Continuous model of HDM-induced AAD. C57BL/6 mice were lightly anesthetized with isoflurane and administered 25 μg lyophilized HDM extract in 50 μl PBS via intranasal (*i.n.)* instillation for up to 11 wks as described in *Materials and Methods*. Black lines indicate days on which HDM was administered. Mice were sacrificed (*sac*) after either 2 wks (short-term exposure), 5 wks (intermediate-term exposure) or 11 wks (long-term exposure). Control groups received i.n. PBS in a similar manner as HDM.

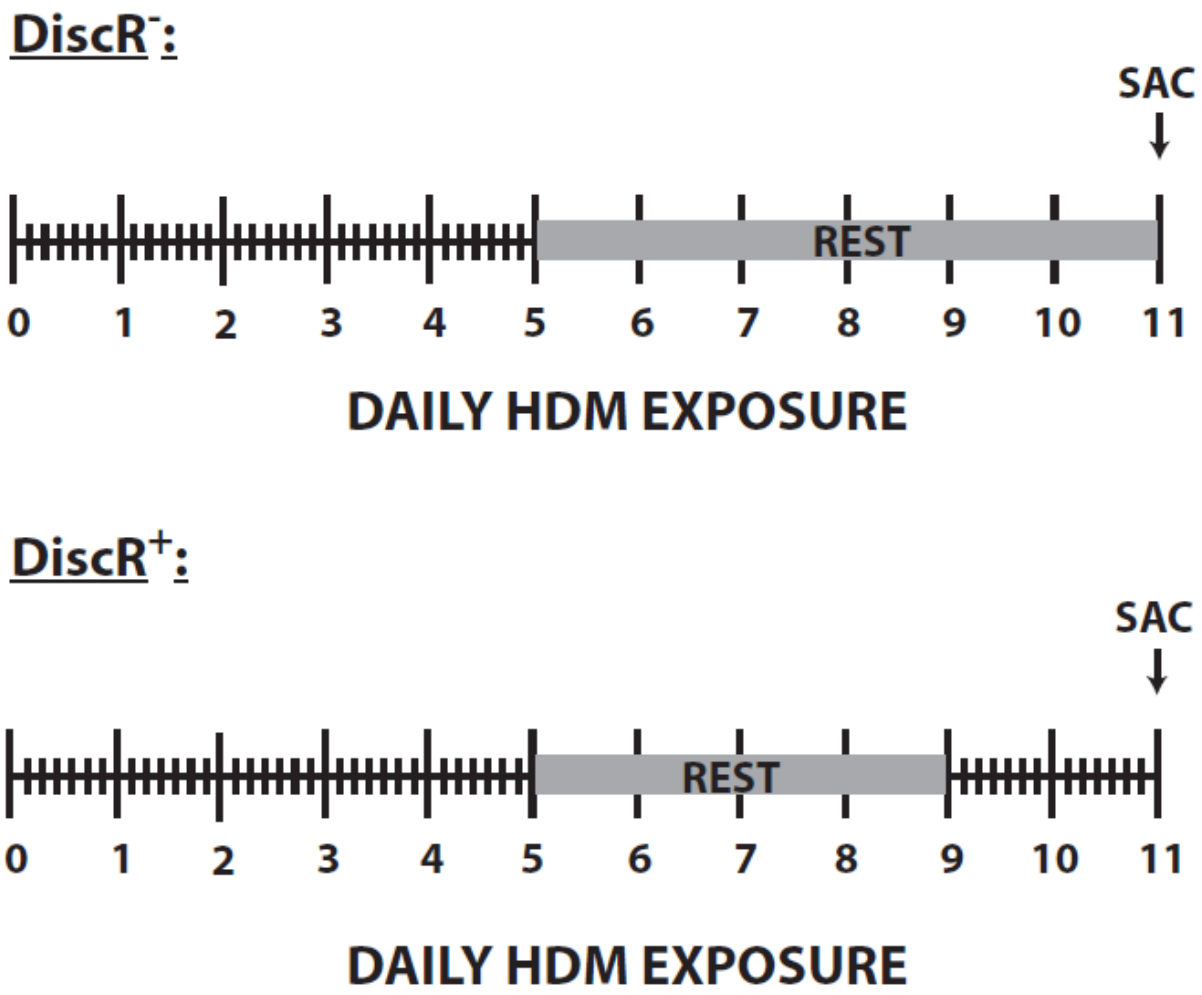

Figure 3. Discontinuous models of HDM-induced AAD. Animals were discontinuously (Disc) administered i.n. HDM with (R^+) or without (R^-) a 2 wk HDM rechallenge. Intervals with tick marks represent weeks when HDM was administered. Shaded intervals indicate rest periods when no HDM was administered.

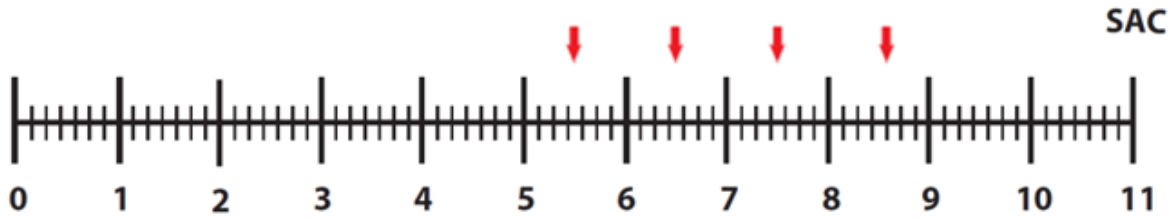

Figure 4. Model of alveolar macrophage depletion. Mice were continuously administered HDM for 11 weeks as shown in Figure 2. Starting at week 5, clodronate or PBS control liposomes were administered i.n. once per week (7 mg/ml; 50 µl per injection) through week 9 to deplete alveolar macrophages (red arrows). Control liposomes were administered at equal volumes in a time matched manner. Animals were sacrificed (sac) post 11 weeks of Ag challenge.

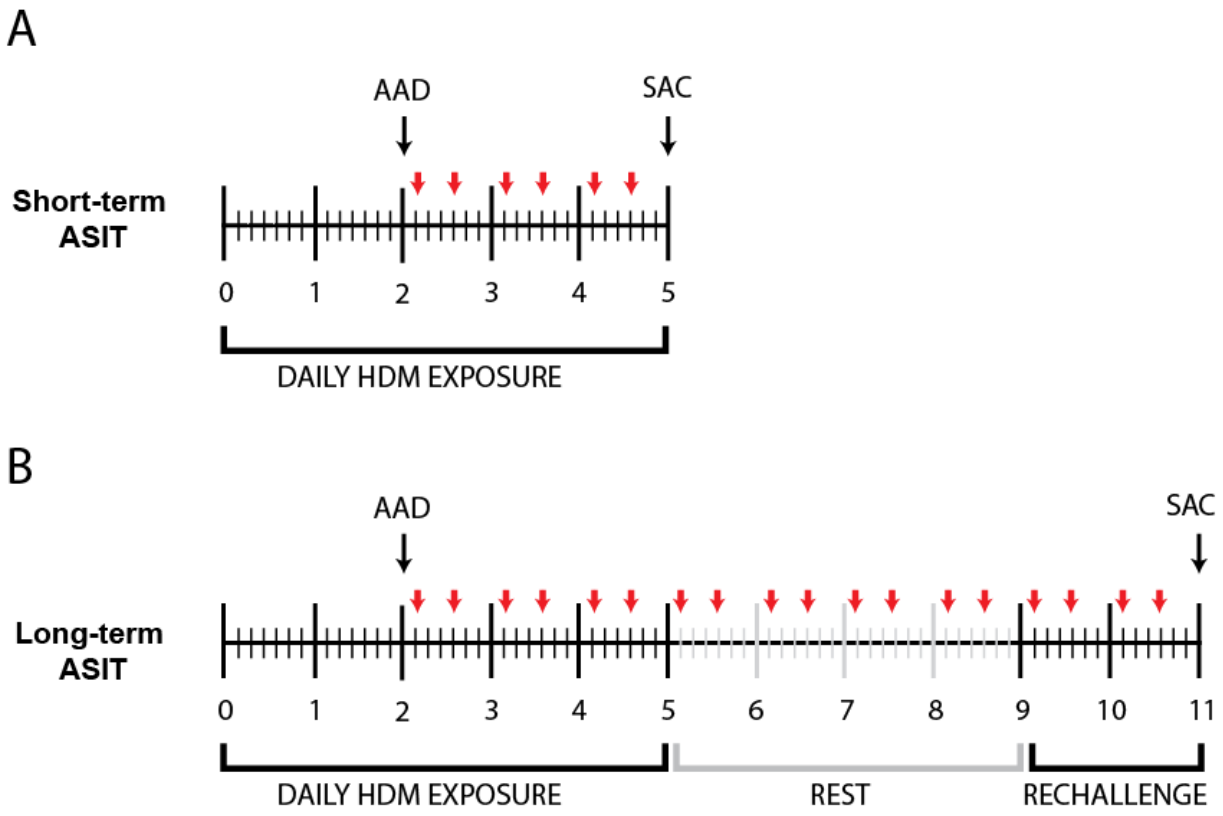

Figure 5. Model of allergen-specific immunotherapy. Animals were challenged with i.n. HDM **(A)** continuously for 5 wks as shown in Figure 2 or **(B)** discontinuously for 11 wks with a 2 wk HDM rechallenge (DiscR^{+}) as shown in Figure 3. Intervals with tick marks represent weeks when HDM was administered. Shaded intervals indicate rest periods when no HDM was administered.
Following 2 weeks of HDM administration, biweekly, subcutaneous ASIT with either low-dose (10 AU) or high-dose (200 AU) HDM extract was administered for either **(A)** 3 wks; total of 6 injections or **(B)** 9 wks; total of 18 injections. Control animals received equal volumes of subcutaneous vehicle (normal saline phenol). Red arrows indicate times of subcutaneous HDM-ASIT administration. All animals were sacrificed (sac) 72 hours post final Ag challenge.

CHAPTER 3

LONG-TERM EXPOSURE TO HOUSE DUST MITE LEADS TO SUPPRESSION OF ALLERGIC AIRWAY DISEASE DESPITE PERSISTENT LUNG INFLAMMATION

Introduction

Allergic asthma is a chronic and debilitating disorder of the airways that impacts nearly 300 million people worldwide. Unfortunately, current pharmacologic therapies for asthma do not specifically alter the underlying immunopathology that contributes to disease and thus have had marginal effects on reducing the overall rate and economic burden of asthma. It is crucial to understand the mechanisms that contribute to the development and suppression of asthma in order to improve upon the standards of care for this widely prevalent and costly disorder.

A vast body of literature documents that asthma results from lack of immunologic tolerance against inhaled, environmental Ags, resulting in uncontrolled Th2 activation, pulmonary eosinophilia, and airway hyper-reactivity (AHR)[158]. We[147] and others[156,159-161] have demonstrated that long term, continuous exposure to an allergen can result in tolerance development and ultimate resolution of allergic airway disease (AAD) in a variety of murine models. Although previous investigations in long-term models of OVA-induced AAD have added tremendously to our knowledge of the tolerogenic mechanisms utilized by the mucosal immune system to suppress allergic inflammation, OVA is far less structurally and

immunologically complex than the majority of human allergens[140]. Therefore, despite their tremendous utility, OVA-induced models of AAD are not likely to involve the entire scope of inflammatory processes that are involved in asthmatic responses. The need persists for more physiologically relevant models of human asthma.

HDM is the most causative human allergen worldwide. It is estimated that 50-85% of all asthmatics harbor an allergy to HDM[37,162], which suggests that the immunomodulatory mechanisms most frequently activated in response to environmental Ags may be impaired in the presence of HDM. Current models of HDM-induced asthma have been quite useful in understanding the multifaceted immune response that is stimulated as a result of short-term Ag exposure[153,163]. Unfortunately, the clinical relevance of these models is limited by the fact that most individuals are exposed to HDM in a long-term, continuous manner due to the ubiquitous nature of this allergen. Furthermore, there is a shortage of literature describing the effects of long-term HDM exposure in the lung. The few studies that have utilized long-term models of HDM-induced AAD have focused heavily on the structural changes that occur in the lung and have not examined the tolerogenic capacity of HDM or its long-term effects on the immune system[154,164].

Given the paucity of data on disease progression in murine models of HDM-induced AAD, the purpose of this study was to investigate the ability of HDM to induce immunologic tolerance with long-term exposure. Our results demonstrated that short-term HDM exposure promoted development of AAD, and long-term HDM exposure promoted suppression of disease, as was evidenced by full resolution of the airway eosinophilia and AHR associated with acute AAD. Suppression of HDM-induced AAD was accompanied by an increase in local $Foxp3^{+}$ regulatory T cells (Treg) and a transition in alveolar macrophages (AM) to an $IL\text{-}10^{+}$ phenotype. We predict that the immunological changes revealed by this novel, biphasic HDM model could offer tremendous insight into clinical strategies for the orchestration of tolerance against human-relevant aeroallergens.

Results

Short- and intermediate-term (2 and 5 wk) HDM exposure resulted in elevated eosinophil levels in the BAL while long-term (11 wk) HDM exposure led to resolution of BAL eosinophilia

In concordance with previous studies that have examined responses to acute HDM instillation using similar models[154], we noted a dramatic (10-fold) increase in total BAL leukocytes after short-term (2 wks) administration when compared to time-matched PBS control animals (*Fig 6A*). Cell counts decreased significantly following intermediate-term (5 wks) HDM administration and remained at a similar level following long-term (11 wks) HDM exposure. However, total BAL leukocytes remained elevated over time-matched control animals following both intermediate- and long-term HDM exposure, indicating persistent inflammation.

As was expected, mice exposed to PBS at all time points harbored few (<3%) eosinophils and neutrophils in the BAL (*Fig 6B*). HDM-exposed mice did not show any elevation in neutrophils above time-matched control animals. However, short- and intermediate-term exposure to HDM resulted in a dramatic increase in eosinophil frequency when compared to time-matched control mice ($p<0.001$). Total number of eosinophils was also significantly elevated after short-term HDM exposure (*Fig 6C*), and although elevated after intermediate-term HDM exposure, did not reach statistical significance due to high variation in eosinophil numbers at this time-point ($35 \pm 8 \times 10^4$). Despite continued elevation of total leukocyte counts, long-term HDM exposure led to a decrease in BAL eosinophil levels that matched frequencies found in control animals (*Fig 6B)*. In addition, BAL eosinophil number decreased significantly following long-term HDM exposure when compared to short-term exposure (*Fig 6C*). This trend was similar to that observed for lymphocytes, which peaked in frequency (*Fig 6B*) and number (*Fig 6C*) after short-term exposure but greatly decreased following long-term HDM exposure. Together, these findings suggest that long-term HDM exposure promotes suppression of allergic inflammation in spite of persistent BAL total leukocyte counts.

In PBS control mice, 95% of BAL cells were macrophages (*Fig 6B*). Macrophage frequency dropped to 28±4 % in short-term HDM mice as a result of a relative increase in eosinophils and lymphocytes (*Fig 6B*). Macrophage frequency rebounded to 88 ± 2% following long-term HDM exposure, a value that did not statistically differ from levels observed in time-matched control mice. However, absolute numbers of macrophages in long-term HDM mice increased 5-fold when compared to time-matched control mice and 2- to 3-fold when compared to short- and intermediate-term HDM mice (*Fig 6C*). Moreover, airway macrophages demonstrated dramatic alterations in their histological appearance over the course of disease (*Fig 6D*). Whereas macrophages from PBS control mice appeared small, round, and inactive (*Fig 6D- upper left*) at all time points, those from short-term HDM (*Fig 6D- upper right*) and intermediate-term (*Fig 6D- lower left*) HDM-exposed mice were more elongated, vacuolated, and began to show evidence of multinucleation, *Fig 6D,E*). Macrophages from long-term HDM mice increased dramatically in size compared to control, short-term HDM, and intermediate-term HDM-exposed macrophages (*Fig 6D- lower right*). In addition, there was a substantial increase in the frequency (*Fig 6E*) and number (*Fig 6F*) of multinucleated macrophages at this time point when compared to those found in control, short-term HDM, and intermediate-term HDM exposed animals, thus indicating that macrophage phenotype was altered over the course of disease.

Serum IgE titers and associated Th2 cytokines were elevated following HDM exposure

Serum levels of HDM-specific IgE were at the lower limit of detection in PBS control animals and appeared to increase steadily over the course of HDM exposure (*Fig 7A*). However, as compared to time-matched PBS controls, significant elevations in HDM-specific IgE were observed only in long-term HDM-exposed mice, which was most likely due to the high variability observed in the more concentrated serum samples. Serum levels of HDM-specific IgE in long-term HDM mice were also significantly elevated over short-term and intermediate-term HDM mice, suggesting ongoing systemic allergic inflammation. HDM-specific IgG_1 levels followed a similar trend as HDM-specific IgE, increasing steadily over the course of HDM exposure and peaking following long-term instillation (*Fig 7B*).

Further evaluation of allergic status was determined via levels of Th2-associated cytokines in HDM-exposed mice. Levels of IL-4 in BAL fluid and lung tissue of short-term HDM-exposed mice were significantly increased over time-matched PBS control animals but decreased following long-term HDM exposure (*Fig 8*). IL-5, which did not appear to be greatly elevated in the BAL fluid of HDM-exposed mice, was, however, significantly increased in the lung tissue with short-term HDM exposure and decreased with long-term HDM exposure. These trends were reversed in the serum; IL-4 and IL-5 appeared to steadily rise over the course of disease, demonstrating additional evidence of ongoing, systemic inflammation. In addition, the pro-regulatory cytokine IL-10 appeared to increase in the serum over time, although no significant differences in IL-10 levels were noted between groups. IL-10 was not detectable in the BAL fluid and detectable only at very low levels in the lung tissue.

Th1 cytokines such as INFγ have been shown to inhibit Th2 cytokine functions and allergic eosinophilia when acting on the airway epithelium[165]. Local (BAL and lung tissue) INFγ levels approximated or fell below the limit of detection in HDM-exposed mice at all time points (*Fig 8*). Intriguingly, INFγ levels appeared to be elevated in the serum of select mice exposed to HDM for either an intermediate- or long-term period. However, these levels were not statistically increased above PBS control mice or short-term HDM mice at either time point due to extremely high variation within groups.

Perivascular and peribronchiolar inflammation was elevated in the lungs of long-term HDM mice despite attenuation of mucus production

As anticipated, PBS control animals demonstrated little to no histological evidence of lung inflammation (*Fig 9A,I*). Early histological alterations were observed after short-term (2 wks) HDM exposure in the form of small, inflammatory pockets (*Fig 9B; short arrow*) and increased following intermediate-term (5 wks) HDM exposure in the form of large perivascular and peribronchiolar inflammatory clusters (*Fig 9C; short arrow*). These clusters were diffuse throughout the majority of the lung lobes and qualitatively consisted of lymphocytes, macrophages, and eosinophils. In addition, the appearance of smooth muscle hypertrophy around the airways was qualitatively evident at this time point

(*Fig 9D; long arrow*). Although BAL eosinophilia was attenuated in long-term HDM mice (*Fig 6B,C*), diffuse peribronchiolar and perivascular inflammation in the lung tissue peaked at this time point (*Fig 9D; short arrow*). Semi-quantitative analysis supported an increase in inflammation above time-matched PBS controls (*Fig 9I*). The composition of this inflammation, however, changed relative to short- and intermediate-term HDM mice such that it was predominantly comprised of macrophages and lymphocytes with few eosinophils present. No further increase in smooth muscle hypertrophy was noted in long-term HDM mice (*Fig 9D; long arrow*). In addition, Mallory's trichrome-stained lung sections did not reveal gross increases in perivascular or peribronchiolar collagen deposition in long-term HDM mice when compared to other stages of the model (*data not shown*).

In PBS control animals, there was no evidence of increased mucus production in smaller airways (*Fig 9E*). Animals exposed to HDM for short-term and intermediate-term periods demonstrated increased mucus production relative to time-matched PBS control mice via positive PAS staining of goblet cells in smaller airways (*Fig 9F,G; arrows*), a conclusion which was supported by blinded scoring results (*Fig 9J*). Despite the presence of large inflammatory aggregates surrounding many airways, mucus production appeared to be attenuated in airways of long-term HDM mice when tissue samples were blindly scored (*Fig 9H; arrows* and *Fig 9J*). Although resolution of airway eosinophilia, decreased Th2 cytokines in the lung compartments, and attenuated airway mucus production suggested that long-term exposure to HDM led to suppression of allergic inflammation, elevated BAL leukocyte counts and peribronchiolar/perivascular inflammation nevertheless persisted at this stage of the model.

Lung resistance was elevated by intermediate-term HDM exposure and was ameliorated by long-term HDM exposure

In order to determine whether resolution of BAL eosinophilia and attenuation of mucus production with long-term (11 wk) HDM exposure correlated with improved airway dynamics, AHR was assessed in response to methacholine challenge in both PBS control and HDM-exposed mice. Baseline respiratory system resistance (Rrs) measurements in PBS mice at the 2 wk, 5 wk, and 11 wk time points

were virtually identical and were thus pooled to obtain a single curve representing all PBS-exposed animals. As expected, Rrs measurements in PBS-exposed mice and all groups of HDM-exposed mice were similar prior to methacholine administration (*Fig 10*). Administration of methacholine led to a dose-dependent increase in Rrs measurements over a range of 1.25 to 100 mg/ml. Compared to PBS mice, short-term (2 wk) HDM mice demonstrated increased resistance in response to intermediate doses of methacholine, although the overall AUC of Rrs did not statistically differ from that of the PBS control cohort. However, intermediate-term (5 wk) HDM mice demonstrated a significant elevation in Rrs when compared to PBS subjects. Rrs for intermediate-term HDM mice was also significantly elevated over short-term HDM mice, suggesting that intermediate-term HDM exposure led to peak airway dysfunction. The increase in AHR at 5 wks was completely diminished with long-term HDM administration, such that Rrs curves in PBS mice and long-term HDM mice did not statistically differ from one another. The correlation between resolution of airway eosinophilia (*Fig 6B,C*) and AHR provided additional evidence that long-term exposure to HDM promoted suppression of AAD despite persistent perivascular/peribronchiolar lung inflammation.

Suppression of AAD with chronic HDM administration was dependent on continuous Ag exposure

Previous studies from our laboratory have demonstrated that resolution of disease following chronic exposure to OVA is dependent on continuous Ag administration[148]. In order to determine whether suppression of AAD occurs in the absence of continuous exposure to HDM, select groups of mice were discontinuously administered HDM for 11 wks as described in Chapter 2, *Fig 3*. Mice that did not receive an HDM rechallenge ($DiscR^-$) following initial discontinuation of HDM showed similar numbers of total BAL leukocytes (*Fig 11A*) and frequency of airway eosinophils (*Fig 11B*) as mice that received continuous HDM for 11 wks. This finding suggested that AAD resolved spontaneously with time in the absence of continued HDM exposure. Of interest, however, the total number of macrophages remained elevated in the $DiscR^-$ mice as in the continuous animals, at a level approximately 9 times higher than that seen in naïve mice.

On the other hand, mice that received a 2 wk HDM rechallenge ($DiscR^+$) following discontinuation of Ag demonstrated a significant increase in total BAL leukocyte counts (*Fig 11A*) when compared to both 11 wk continuous and $DiscR^-$ mice. Macrophage frequency decreased significantly when compared to continuously exposed mice (*Fig 11B*). Additionally, $DiscR^+$ mice demonstrated a significant increase in eosinophil frequency when compared to 11 wk continuous mice (*Fig 11B*). Despite the fact that eosinophil frequency increased significantly in $DiscR^+$ mice when compared to 11 wk continuous mice, levels reached only 21 ± 4% following 2 wk HDM rechallenge, half of that observed in naïve mice subjected to short-term (2 wks) HDM exposure (*Fig 6B*). Together, these data suggested that although continuous Ag exposure was required to actively suppress BAL eosinophilia, prior Ag exposure may attenuate severity of allergic inflammation during re-exposure to the triggering allergen.

Attenuated disease in $DiscR^+$ mice when compared to short-term HDM mice suggests that T cells may become exhausted with prolonged courses of HDM exposure. To determine whether the tolerant phenotype observed with long-term HDM exposure could be attributed to exhaustion, expression of programmed death 1 (PD-1), whose high expression on T cells is associated with exhaustion, was examined on $CD4^+$ T cells in naïve, short-term HDM, and long-term HDM mice. When compared to naïve mice, frequency of PD-1^{hi} $CD4^+$ T cells in short-term mice were significantly increased in all three lung compartments (*Fig 12*). However, this frequency was not increased in long-term HDM mice when compared to short-term HDM mice, suggesting that T cells were not further exhausted with prolonged HDM exposure.

Suppression of AAD with long-term HDM instillation correlated with an increase in regulatory leukocytes at the site of inflammation

Previous data from our laboratory have shown that key interactions facilitating tolerance to inhaled Ags occur at the site of inflammation[150,152]. As a result, we examined percentages and total numbers of $Foxp3^+$ Tregs in the lung compartments (BAL, HLN, and lung tissue) over an 11-wk course of HDM exposure. When compared with time-matched PBS control mice, short-term (2 wk) HDM-

exposed mice demonstrated a significant increase in the frequency of Foxp3$^+$ T cells relative to total CD4$^+$ T cells in the BAL and lung tissue, while intermediate-term HDM-exposed mice showed an increased Foxp3$^+$ Treg frequency solely in the latter compartment (*Fig 13A,B*). Foxp3$^+$ Treg frequency in the BAL and lung tissue was not further elevated in long-term (11 wk) HDM-exposed mice when compared to short- or intermediate-term HDM-exposed animals. Total numbers of Foxp3$^+$ Tregs in the BAL and lung tissue were significantly increased over PBS control mice at each time point due to general increases in total leukocyte counts with HDM exposure (*Fig 6A*) but were not increased in long-term HDM mice relative to short-term or intermediate-term HDM mice (*Fig 13C*).

Foxp3$^+$ Treg frequency among total CD4+ T cells in the HLN was approximately 14 ± 2% in PBS control groups and was not significantly elevated with short-term or intermediate-term HDM exposure (*Fig 13A,B*). However, long-term HDM-exposed mice demonstrated significant increases in HLN Foxp3$^+$ Treg frequency (20 ± 2%) when compared to time-matched PBS control, short-term HDM and intermediate-term HDM animals. In contrast, total numbers of Foxp3+ Tregs in the HLN demonstrated steady expansion over the course of HDM exposure, with significant elevations in intermediate-term HDM animals over short-term HDM mice and in long-term HDM mice over both short-term and intermediate-term animals (*Fig 13B*). Most intriguingly, these kinetics correlated with resolution of eosinophilia in the airways (*Fig 6B,C*). Of note, no major differences were observed in the frequency of total CD4$^+$ T cells in the HLN across the various stages of the model (*Fig 14*). These findings suggest that selective expansion of Tregs in the HLN may play a critical role in suppression of AAD following long-term Ag exposure.

In addition to T regulatory cells, there is growing evidence that M2 macrophages can exert regulatory effects in the context of asthma. Because alveolar macrophages (AMs) are the predominant cell population within the naïve airway and because they increased in frequency and number in long-term HDM mice (*Fig 6B,C*), we examined changes in M2 alveolar macrophage status (F4/80$^+$CD11c$^+$ cells; *Fig 15A*) throughout the course of the model. Production of IL-10 (a hallmark regulatory cytokine produced by M2 macrophages[75]) was examined by administering HDM or time-matched PBS control to

IL-10-GFP reporter (Vert-x) mice both short-term (2 wks) and long-term (11 wks) as described in *Fig 2*. While neither PBS nor short-term HDM administration increased IL-10 production by AMs in the BAL or lung tissue when compared to negative control animals, AMs from long-term HDM mice demonstrated a rightward shift in GFP expression indicative of elevated IL-10 production (*Fig 15B*). This shift represented a significant increase in mean fluorescence intensity (MFI) of IL-10-GFP in long-term HDM mice relative to time-matched PBS animals and short-term HDM animals in both BAL and lung tissue (*Fig 15C*). Frequency of IL-10^+ AMs in short-term HDM exposed to HDM was not increased over levels observed in time-matched PBS animals in the BAL and lung tissue (*Fig 15D*). However, frequency of IL-10^+ AMs was significantly increased over time-matched PBS animals and short-term HDM animals in long-term HDM mice. Of note, no differences in frequency of IL-10^+ macrophages were observed between PBS animals at either time point, demonstrating that induction of IL-10^+ macrophages was specific to HDM administration. Quite interestingly, induction of IL-10^+ macrophages with long-term HDM administration was observed only in F4/80^+CD$11c^+$ AMs and not in F4/80^+CD$11c^-$ interstitial lung macrophages (IMs; *Fig 15E*). Forward and side scatter analysis of IL-10^+ AMs demonstrated that these cells were large in size and highly granular (*data not shown*), thus correlating with the appearance of big, densely staining macrophages in the BAL after 11 wks HDM administration (*Fig 6D*).

Because disease severity was less pronounced in DiscR^+ mice when compared to acute (2 wk) HDM mice, we examined whether AMs adopted an IL-10^+ M2 phenotype in mice that were discontinuously administered HDM. AMs in DiscR^+ mice produced similar levels of IL-10 in the BAL as AMs from mice administered HDM continuously for 11 wks (*Fig 16*). Furthermore, both 11 wk continuous and DiscR^+ AMs in the BAL produced significantly more IL-10 than AMs in DiscR^- mice ($p<0.001$). However, neither DiscR^- or DiscR^+ AMs in the lung produced much IL-10 when compared to 11 wk continuous AMs.

Discussion

The allergic response to inhaled HDM has been extensively characterized in its acute stages; however, few studies have examined the effects of long-term, inhaled HDM exposure on the inflammatory response. The present findings demonstrate that inhaled HDM exposure stimulated a biphasic response, in which short-term (2 wk) and intermediate-term (5 wk) intranasal exposure incited hallmarks of AAD (elevated BAL eosinophilia, mucus production, and AHR upon methacholine challenge) and long-term (11 wk) exposure promoted suppression of allergic inflammation (decreased BAL eosinophilia and mucus production with lack of AHR). Interestingly, long-term HDM mice maintained persistent perivascular/peribronchiolar, mononuclear cell inflammation within the lungs.

Our laboratory has previously demonstrated that continuous aerosol administration of the model Ag OVA leads to a biphasic response in which short-term exposure promotes development of AAD that resolves following long-term exposure[147]. Long-term HDM administration showed a similar pattern to the long-term OVA model: suppression of AAD with decreased BAL eosinophilia, decreased mucus production, and resolution of AHR upon methacholine challenge. In support of our findings, others have also observed attenuation of BAL eosinophilia during long-term HDM exposure; however, this observation has previously been noted in conjunction with increased airway neutrophilia and significant vascular remodeling[164,166,167], indicators of progressive AAD rather than attenuated AAD. The reason for this discrepancy between models is not entirely known, but one might speculate that this could be a consequence of strain differences, as the previously referenced studies were all performed in BALB/c rather than C57BL/6 mice. It has, in fact, been shown that BALB/c mice exposed intratracheally to the fungal allergen *Aspergillus fumigatus* demonstrate a neutrophil predominance when compared with C57BL/6 mice due to augmented TNFα production by dendritic cells and macrophages[168]. Moreover, TNFα has been shown to influence vascular and lymphatic remodeling in mice with sustained airway inflammation[169]. In addition to strain, dose and timing of HDM exposure are likely to affect the phenotype observed in this model. While it is well-established that tolerance development generally

requires long-term allergen challenge, the dose of HDM required for tolerance generation is a trickier matter, as tolerance to long-term HDM exposure has not previously been demonstrated. While others have shown limited dependency of allergen dose on pathology, cytokine responses, and AHR in short-term, HDM-exposed mice[170], dose effects on long-term HDM exposure have not been well characterized and require appropriate follow-up studies.

Although the effects of long-term OVA exposure on suppression of methacholine-induced AHR are well established, this study is the first to relate resolution of eosinophilia with concurrent suppression of AHR in an HDM model. The reason for suppression of AHR with long-term HDM challenge is not definitively known, and it is not guaranteed that the reduction in eosinophils had a causal role in this response[171]. Although one group has shown that long-term (20 weeks) HDM exposure does not result in resolution of AHR[166], in their study, HDM was administered only three times per week as opposed to five. Thus, continuous Ag instillation (a hallmark of both our OVA and HDM protocols) may be required for tolerance induction and subsequent suppression of AAD. In addition, a causative role between mucus overproduction and development of AHR has been described[172]. Relative to short-term and intermediate-term HDM-exposed animals, long-term HDM mice demonstrated a noticeable reduction in mucus within the airways. This reduction in mucus may have directly contributed to the resolution of AHR observed with long-term HDM exposure.

Despite the relative reduction in local inflammatory responses observed in long-term HDM mice when compared to short-term HDM mice, the former group demonstrated higher HDM-specific IgE levels and Th2 cytokines systemically. Persistent (or even increased) systemic IgE responses with long-term, inhalational Ag exposure has been previously demonstrated by our laboratory[148] as well as by others[156]. Moreover, our previous findings indicate that mice that develop tolerance with long-term OVA exposure demonstrate greater subcutaneous late phase responses after OVA footpad injection than short-term OVA-exposed mice[149]. Thus, it appears that development of tolerance to inhaled allergens with long-term exposure is a localized response that is not dependent upon suppression of systemic inflammation.

Nevertheless, trending increases in IL-4 and IL-5 in the serum of long-term HDM mice was matched by increased IL-10 levels, suggesting some degree of systemic immunoregulation as well. Additionally, systemic HDM-specific IgG_1 responses were also increased over the course of Ag exposure and were significantly elevated in long-term HDM mice relative to short- and intermediate-term HDM mice. Successful courses of allergen-specific immunotherapy (the only etiological therapy for treatment of asthma and allergy) is commonly associated with increases in IgG_1 (murine equivalent of IgG4 in humans) in both mice[122] and humans[173]. Murine IgG_1/human IgG_4 have been thought to act as blocking antibodies that can compete with IgE for allergen binding. Thus, systemic increases in IgG_1 with long-term HDM exposure may serve as an additional indication of tolerance development within this model. Successful courses of allergen-specific immunotherapy have also been associated with immunodeviation, during which T helper cells shift from a predominantly Th2 to Th1 phenotype[174]. Although Th2 cytokines appeared to decrease locally following long-term HDM exposure, we did not observe any evidence that such a shift occurs within our model. INFγ levels remained at or below the limit of detection in local (BAL and lung tissue) compartments. Although INFγ levels appeared to be elevated in the serum with intermediate-term and long-term HDM exposure, they were associated with extremely large variations within groups. Thus, this data suggests that a switch from Th2 to a Th1 phenotype is not likely to explain suppression of AAD with long-term HDM exposure.

Most intriguingly, the inflammatory pattern associated with long-term HDM instillation demonstrated some key differences from that associated with long-term OVA administration. The time course of AAD induction and resolution differed, with peak AAD occurring after 7-10 days of airway exposure to OVA and resolution developing after 6 wks of exposure[147]. A longer time course was needed for HDM, with peak AAD seen after 5 wks of exposure and resolution of the AAD responses requiring 11 wks. Moreover, long-term OVA administration is marked by full resolution of perivascular and peribronchiolar inflammation in the lung tissue, a decrease in total BAL leukocyte counts to baseline levels, and little evidence of airway remodeling[147,175]. In contrast, mice exposed to long-term HDM (11

wks) had persistent inflammation in their BAL and lung tissue with some evidence of airway remodeling (in the form of smooth muscle hypertrophy). While smooth muscle hypertrophy was qualitatively increased in long-term HDM mice relative to short-term HDM mice, little difference was noted when compared to airway smooth muscle in intermediate-term HDM mice. These findings are supported by previous work, which demonstrates that airway smooth muscle mass increases with 5 wks of HDM exposure but does not further enlarge with 10 wks of HDM exposure[176]. However, in our model, the inflammatory composition in long-term HDM mice changed from eosinophilic (2 and 5 wks) to mononuclear (11 wks) in nature. Persistent mononuclear inflammation in spite of attenuated mucus production and AHR may be attributed to the complexity of HDM, which is comprised of numerous immunogenic compounds with various biological properties, including LPS, β-glucans, chitin, and enzymatically-active proteases[141,177].

The role of LPS in the pathogenesis and regulation of allergic responses has long been of interest to the scientific community. TLR4 signaling from contaminating LPS in HDM extract has been clearly confirmed to contribute to the development of HDM allergy, particularly with regards to mucosal sensitization and initiation of Th2 polarization[8]. Yet despite the fact that mice are continuously exposed to LPS within this model, neutrophils (a key responder in LPS-mediated inflammation) are not significantly elevated at any observed time point following HDM exposure. As has been previously demonstrated, it is likely that neutrophils were only transiently elevated within the BAL for hours following initial allergen challenge, after which time they were replaced by eosinophils[178]. Thus, LPS is not likely to have had a critical role in the phenotypes observed within later stages of this model. Moreover, we have previously demonstrated that chronic inhalation of LPS in an aerosol solution is not required for development of immune tolerance to long-term OVA exposure, which is especially noteworthy because our OVA and HDM extracts have similar LPS contents[179]. Nevertheless, OVA is a simpler Ag than HDM in all other regards. The numerous compounds in HDM may induce innate immune responses by binding protease-activation receptors (PARs), Toll-like receptors (TLRs), or C-type lectin receptors (CTRs) in innate

immune cells such as macrophages and mast cells[180] and in airway epithelial cells[181]. It is possible that continued activation of these innate pathways resulted in persistent inflammation and smooth muscle hypertrophy in the long-term HDM model despite apparent development of airway tolerance to HDM. Persistence of inflammation with long-term HDM exposure could represent ongoing, non-eosinophilic asthma, analogous to the paucigranulocytic asthma seen in a third of adults with asthma, including subjects with HDM sensitivity[182]. However, paucigranulocytic asthmatics demonstrate hyperresponsiveness to methacholine[182] – unlike the resolution of AHR seen in our long-term HDM animals. Thus, long-term HDM-exposed mice appeared to have regained respiratory tolerance to HDM, perhaps analogous to the 60-70% of childhood asthmatics in whom asthma remits by puberty[183-185]. In this regard, it is intriguing to speculate that potential subclinical lung inflammation (in the absence of airway eosinophilia, methacholine AHR, or asthma symptoms) could play a role in the relapse of asthma in 40-50% of remitted individuals in later adulthood[183-185].

Respiratory tolerance to common aeroallergens has been shown to involve a variety of leukocyte populations, the most established of which is the Foxp3$^+$ Treg. Studies have shown that patients with asthma have a deficiency in Foxp3$^+$ Treg levels[49,50] and that induction of Tregs (e.g. through corticosteroid use and immunotherapy) can play a crucial role in altering the progression of allergy and asthma[186,187]. Foxp3$^+$ Treg frequency was elevated in the HLN specifically with long-term but not short-term or intermediate-term exposure, a finding that has not been reported in previous studies that have administered HDM for long-term periods. Intriguingly, absolute numbers of Foxp3$^+$ Tregs in the HLN (but not in the BAL or lung tissue) demonstrated steady expansion over the course of HDM exposure. Moreover, the kinetics of Treg proliferation occurred prior to suppression of disease, suggesting a causal role for HLN Tregs in tolerance development. Previous findings from our OVA model demonstrate that Treg accumulation in the HLN correlates with development of immune tolerance[150] and that this induction occurs specifically through interactions with B regulatory cells[152]. Thus, increases in HLN Tregs with long-term HDM exposure may have played an important role in the suppression of AAD. Of

note, T cell exhaustion is not likely to have contributed to the phenotype observed with long-term HDM exposure since the frequency of PD-1^{hi} CD4^{+} T cells in all three lung compartments was not increased at this time point when compared to short-term HDM mice; however, this possibility should be more definitely ruled out via lymphoproliferative assays and examination of other exhaustion markers in combination with PD-1 (e.g. LAG-3).

In addition to increased Tregs, long-term HDM-exposed BAL and lungs showed elevations in macrophage numbers. These macrophages were morphologically distinct from those observed at other stages of the model in that they were larger in size and often multinucleated, suggesting that they may exhibit different functional characteristics. Other studies have also demonstrated that long-term dosing regimens with HDM extract leads to the formation of large, activated, multinucleated macrophages in the lungs, although these macrophages have not previously been further characterized[188]. Macrophages that undergo fusion with other macrophages to form multinucleated cells are a prominent feature of some chronic inflammatory states in the lung, including sarcoidosis and hypersensitivity pneumonitis. On the other hand, multinucleated giant cells have also been implicated in the clearance of lung eosinophils through phagocytosis[189], which may explain the disappearance of eosinophils in chronic HDM mice. Macrophage fusion is enhanced by factors that contribute to the generation of an M2 phenotype, including IL-4 and IL-13[190,191]. Additionally, GM-CSF has been shown to promote AM differentiation into multinucleated macrophages[192], and HDM-induced AAD has been shown to be dependent on endogenous GM-CSF production[153]. Further studies in IL-10-GFP reporter mice demonstrated that AMs in BAL and lung tissue of long-term HDM mice exhibited an enhanced ability to produce the anti-inflammatory cytokine IL-10 relative to AMs in both short-term HDM-exposed mice and time-matched PBS control animals. It is currently undetermined as to why long-term exposure to HDM would induce this phenotypic switch in macrophages, although it has been postulated that chronic TLR4 signaling through LPS exposure may stimulate this response as a feedback mechanism for curtailing inflammation[193]. However, we have not observed multinucleated giant AMs in our long-term OVA model, suggesting that

LPS alone is not responsible for this phenotypic switch. Interestingly, enhanced production of IL-10 with long-term HDM exposure was found exclusively in AMs and not IMs. This observation directly contrasts with previous evidence that suggests IMs, and not AMs, assume an IL-10^+ phenotype upon exposure to an allergen in the presence of LPS[102]. Intriguingly, studies have demonstrated that IL-10^+ macrophages are critical for the maintenance of Foxp3 expression in mucosal Tregs[105]. Thus, it is possible that IL-10^+ AMs from long-term HDM mice may have exerted indirect suppressive effects on AAD through their Treg-enhancing activity. IL-10 has been shown to play an important role in regulating the severity of AAD[194,195] and is often decreased in the BAL of asthmatic patients relative to healthy controls[63]. Moreover, it has been shown that in the setting of asthma, AMs have a reduced capacity to produce IL-10, and that inhaled steroids used for asthma treatment can increase AM capacity to express IL-10[196]. However, it remains to be known whether the IL-10^+ AMs induced by chronic HDM exposure are indeed active contributors to disease suppression or simply markers of a tolerogenic lung environment. This question will be further explored in Chapter 4.

As we have observed in the OVA model, disease suppression in this HDM-induced model of AAD was dependent upon continuous administration of Ag[148]. Thus, when $DiscR^+$ mice were subsequently rechallenged with HDM, BAL eosinophilia was elevated when compared to levels in continuous and $DiscR^-$ mice. However, the fact that frequency of eosinophils in the BAL of $DiscR^+$ mice was only half of that observed after 2 wk HDM challenge in naïve mice suggested that prior exposure to HDM attenuated disease severity upon restimulation. These findings may be pertinent to the concept of allergen-specific immunotherapy (ASIT), an etiological therapy for asthma in which repeated administration of the triggering allergen decreases the threshold of reactivity to this causative substance. Multiple studies have demonstrated that ASIT confirms many of its immunological benefits for years even after discontinuation of therapy[115,116], although the exact reasons for this are still unknown. Based on this observation, it would seem likely that $DiscR^+$ mice exposed to a 2 wk HDM rechallenge may have had more opportunity for tolerance development during the initial 5 wk HDM exposure period than naïve

mice exposed to 2 wks of HDM, and in turn the former population may have been less prone to developing BAL eosinophilia than the latter. This speculation was supported by the finding that AMs in the BAL of DiscR^{+} mice produced similar levels of IL-10 as AMs in 11 wk continuous mice, whereas AMs from 2 wk HDM mice did not. Interestingly, ASIT has been shown to enhance IL-10 production in tissue-resident macrophages[130].

In summary, the aforementioned data provides the first evidence that continuous, long-term HDM exposure leads to suppression of AAD associated with short- and intermediate-term HDM exposure, including resolution of eosinophilia and AHR. Furthermore, suppression of AAD coincides with increases in Foxp3^{+} Tregs in the HLN, a finding previously observed with long-term OVA exposure but never before reported for HDM. However, fundamental differences from the OVA model were noted with long-term HDM instillation, including persistent mononuclear inflammation in the peribronchiolar/perivascular regions of the lung despite abrogation of AHR as well as the formation of IL-10^{+} AMs. We believe that results from this long-term HDM model are more clinically representative of the processes involved with tolerance development in human subjects. Thus, this model has tremendous utility for exploring the mechanisms governing immune regulation against complex aeroallergens. Furthermore, continued investigation into this clinically-relevant, biphasic mouse model of AAD may subsequently lead to more successful approaches for tolerance induction in individuals with asthma.

Figures

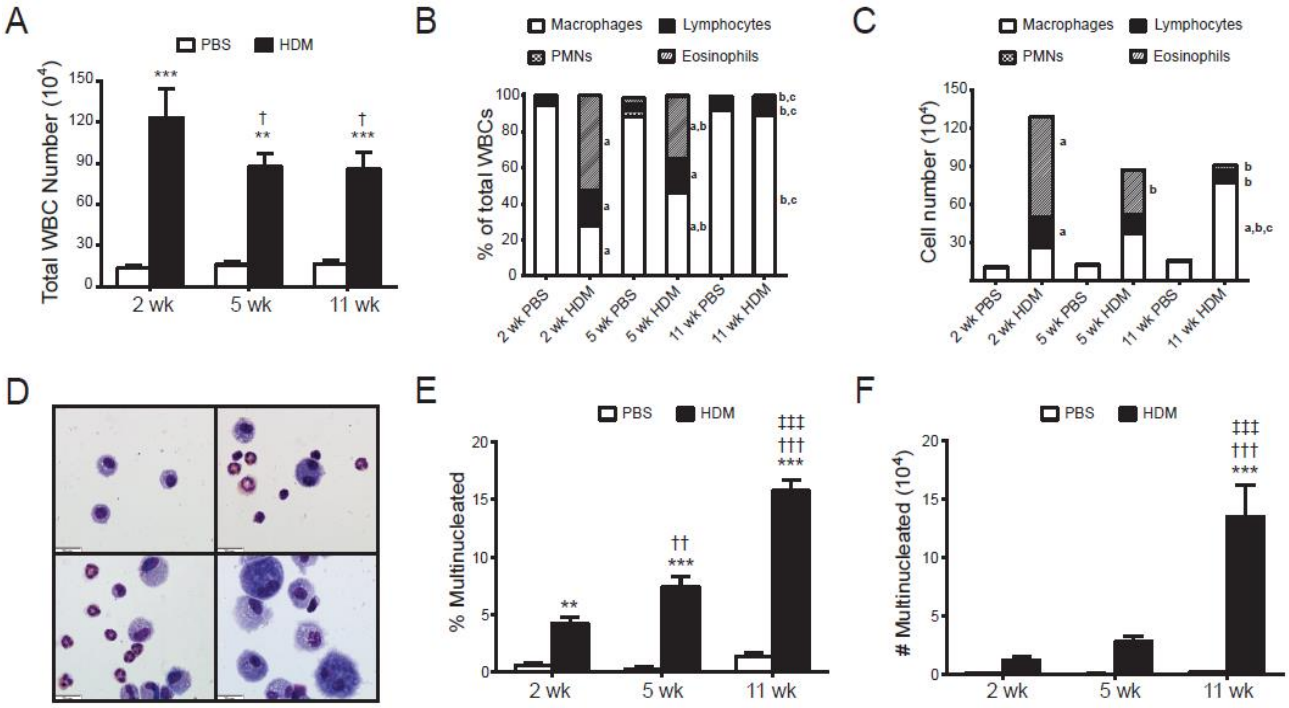

Figure 6. BAL eosinophilia peaks with short-term HDM exposure and resolves with long-term HDM exposure. Female C57BL/6 mice were exposed i.n. to PBS or HDM extract for up to 11 wks as described in Chapter 2, *Fig 2*. **(A)** At sacrifice, total BAL leukocytes were harvested and manually counted on a hemocytometer. **(B-D)** Cytocentrifuged preparations were stained with May-Grünwald Giemsa and differential analysis was manually performed. For (D), the four panels represent: short-term PBS control (upper left); short-term HDM (upper right); intermediate-term HDM (lower left); long-term HDM (lower right). X60, scale bar = 20 μm. (**E-F**) Further analysis was performed on macrophage populations from (D) and percentage (E) and number (F) of multinucleated versus mononucleated macrophages were manually determined. Values for (E) are shown as a percentage of multinucleated macrophages out of total macrophages. Data represent mean ± SEM values; n = 8-13 per group (PBS; values pooled from 2-3 independent experiments), 16-21 per group (HDM; values pooled from 3-5 independent experiments). **(A, E-F)** **p<0.01, ***p<0.001 vs time-matched PBS control; †p < 0.05, ††p<0.01, †††p<0.001 vs. short-

term HDM; ‡‡‡$p<0.001$ vs. intermediate-term HDM **(B,C)** [a]$p<0.05$ vs. time-matched PBS control; [b]$p<0.05$ vs. short-term HDM; [C]$p<0.05$ vs. intermediate-term HDM.

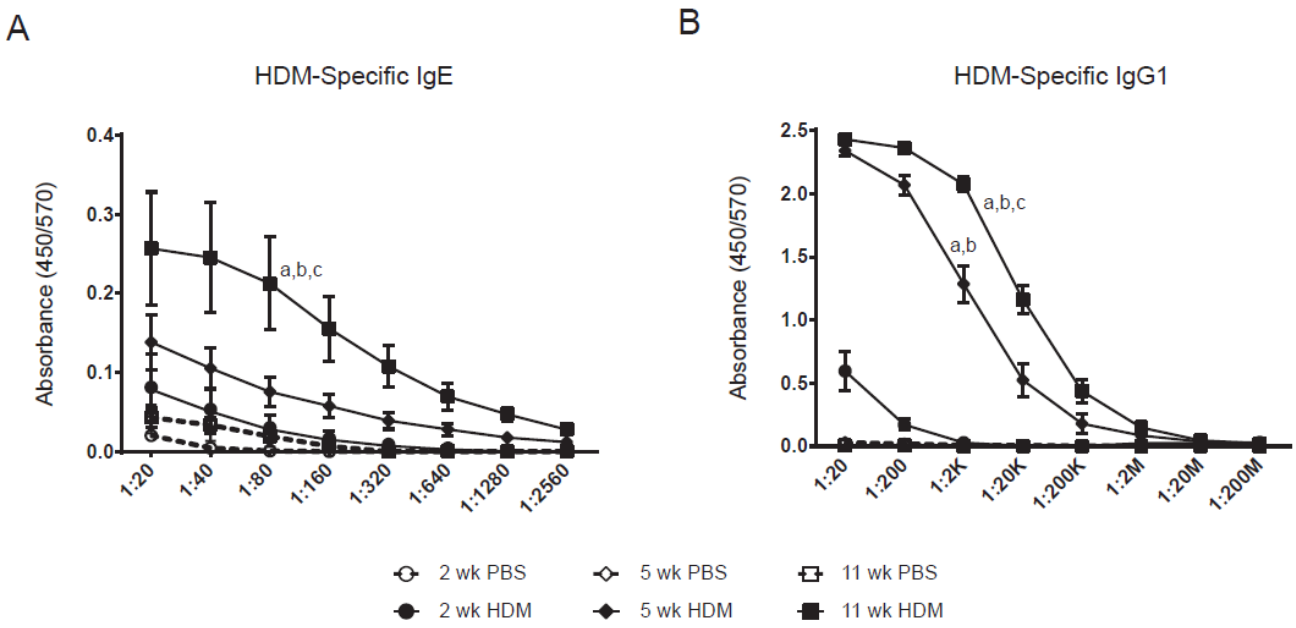

Figure 7. HDM-specific IgE and IgG1 gradually increase over the course of HDM exposure. At sacrifice, serum was collected from both naïve and HDM-exposed animals. HDM-specific **(A)** IgE and **(B)** IgG1 levels were determined via ELISA. Dual absorbance at 450 nm and 570 nm was calculated over a range of dilutions. Data represent mean ± SEM values; n = 6-8 mice per group. For statistical purposes, group comparisons were based on area under the curve (AUC) measurements. [a]$p<0.01$ vs. AUC of time-matched PBS control; [b]$p<0.01$ vs. AUC of short-term HDM; [c]$p<0.01$ vs. AUC of intermediate-term HDM. K, thousand; M, million.

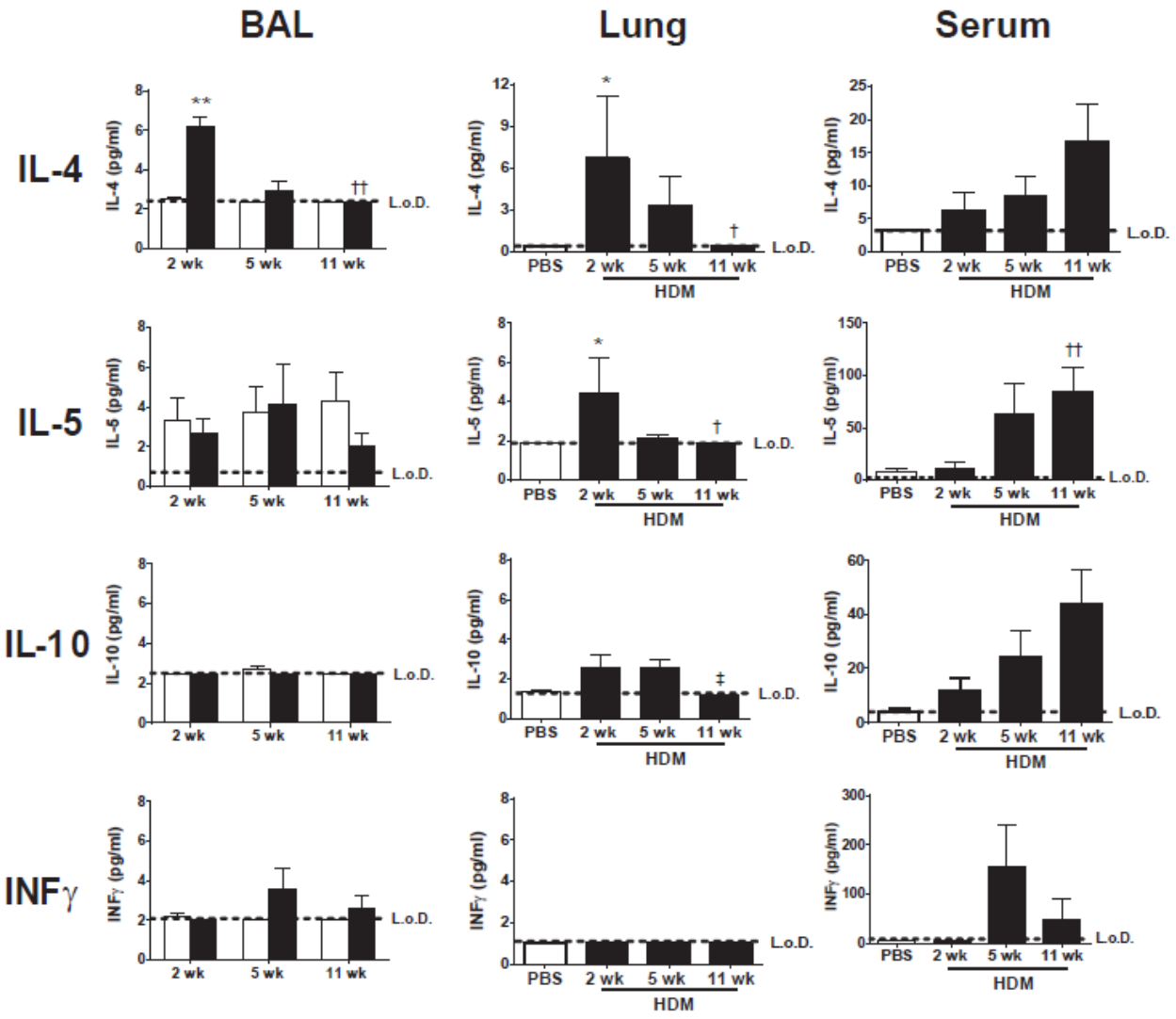

Figure 8. Th2 cytokines peak in the lung compartments following short-term HDM exposure. At sacrifice, BAL supernatant was collected from PBS-exposed (unshaded bars) and HDM-exposed (shaded bars) C57BL/6 females and concentrated ten-fold. Lung tissue homogenates and serum were collected from PBS (short-term only) and HDM animals. Cytokine levels were determined via multiplex analysis. Data represent mean ± SEM values; n = 5 per group (BAL), 3-4 per group (lung), 3 (serum; PBS), 9-10 per group (serum; HDM). *p<0.05, **p <0.01 vs. PBS control; †p<0.05, ††p<0.01 vs. short-term HDM; ‡ p<0.05 vs. intermediate-term HDM. L.o.D; limit of detection.

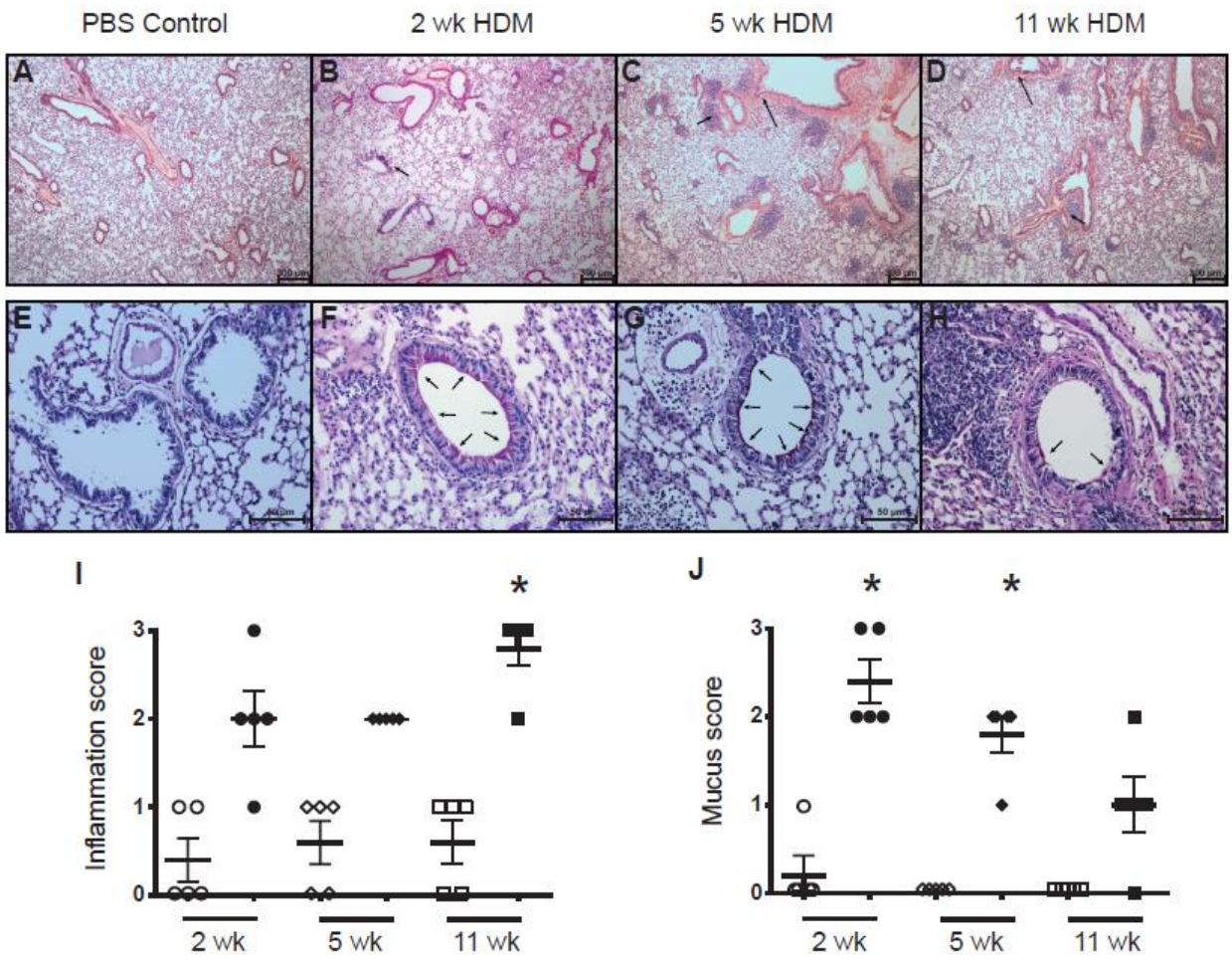

Figure 9. HDM-exposed mice demonstrate persistent perivascular/peribronchiolar inflammation. Formalin-fixed lungs were processed in a standard manner and stained with H&E and PAS. (**Top Row; A-D)** H&E (X4, scale bar = 300 μm). Short arrows highlight examples of perivascular/peribronchiolar inflammation. Long arrows highlight examples of smooth muscle hypertrophy. (**Bottom Row; E-H)** PAS (X20, scale bar = 50 μm). Arrows highlight mucus production by airway goblet cells. **(I)** Inflammation scores and **(J)** mucus scores were determined in a blinded fashion on a severity scale from 0-3 for PBS control (open shapes) and HDM (closed shapes) mice. Data represent mean ± SEM values; n = 5 per group. *p<0.05 vs. time-matched PBS control.

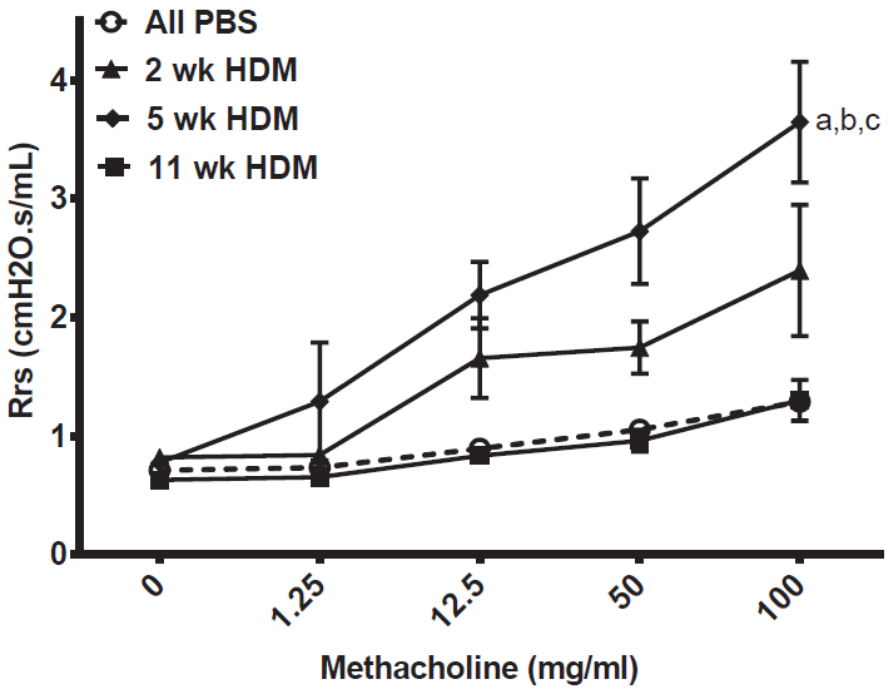

Figure 10. AHR peaks following intermediate-term HDM exposure but is suppressed with long-term HDM exposure. C57BL/6 females underwent tracheostomy and were mechanically ventilated prior to methacholine exposure. Changes in Rrs responses to increasing doses of aerosolized methacholine were determined using the flexiVent system (SCIREQ). Since no differences were observed within AHR measurements for PBS control animals at 2, 5, or 11 wks, AHR data from PBS control mice were pooled to generate a single curve (All PBS). Data represent mean ± SEM values; n =4-5 per group (HDM), 8 (All PBS). For statistical purposes, group comparisons were based on area under the curve (AUC) measurements. [a]$p<0.001$ vs. All PBS AUC; [b]$p<0.001$ vs. long-term HDM AUC; [c]$p<0.01$ vs. short-term HDM AUC.

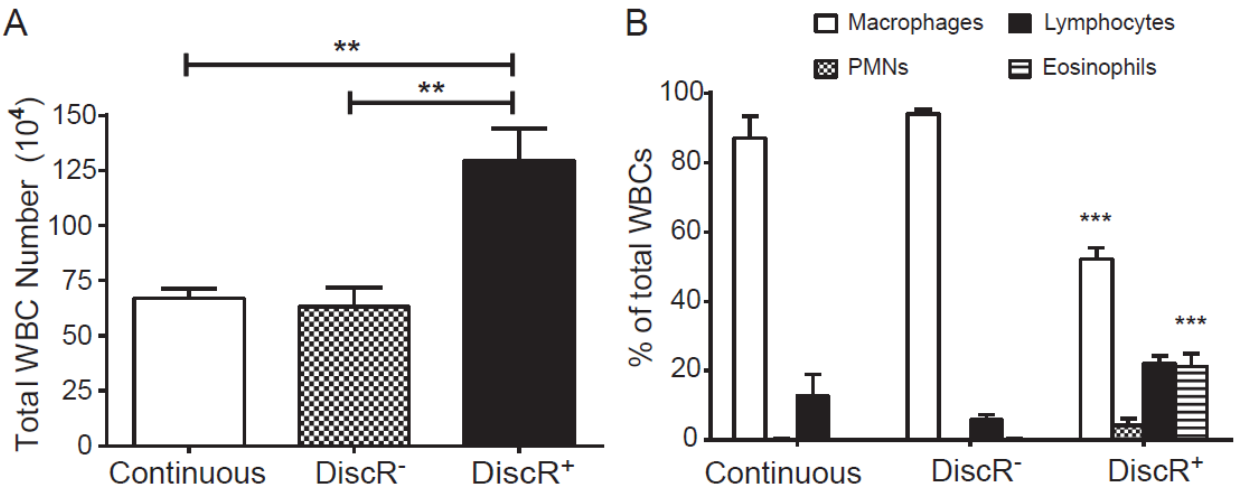

Figure 11. Interruption of continuous HDM exposure leads to reappearance of BAL eosinophilia. Mice were exposed to 11 wks HDM in a continuous or discontinuous manner as described in Fig 3. Briefly, discontinuously exposed mice (Disc) were given 5 wks of continuous, i.n. HDM, after which time mice were either rested for the duration of the 11 wk period (R^-) or rested for 4 wks prior to a 2 wk HDM rechallenge (R^+). **(A)** At sacrifice, total leukocytes were harvested from BAL and manually counted on a hemocytometer (**B**) Differentials were manually determined using cytocentrifuged preparations stained with May-Grünwald Giemsa. Data represent mean ± SEM values; n = 5 mice per group. **(A)** **$p<0.01$ **(B)** ***$p<0.001$ vs continuous and $DiscR^-$ mice.

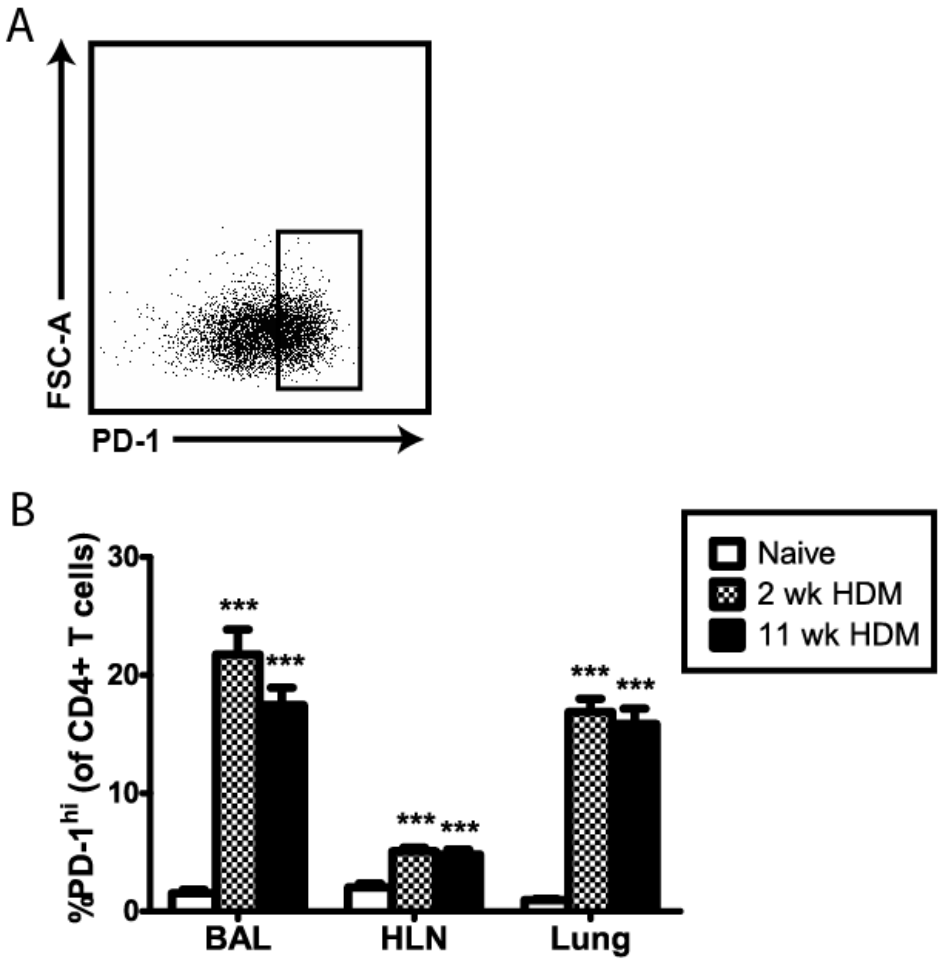

Figure 12. $CD4^+$ T cell exhaustion does not increase in long-term HDM mice when compared to short-term HDM mice. C57BL/6 mice were exposed to short-term or long-term HDM as shown in Fig 2, or not exposed to Ag (naïve). **(A)** Sample FACS plot demonstrating gating for $PD\text{-}1^{hi}$ T cells among the population of $CD4^+$ T cells in the lung. **(B)** Levels of $PD\text{-}1^{hi}$ $CD4^+$ T cells were calculated as a percentage of all $CD4^+$ T cells. Data represent mean ± SEM values; n = 6-10 mice per group. ***$p<0.001$ vs naïve.

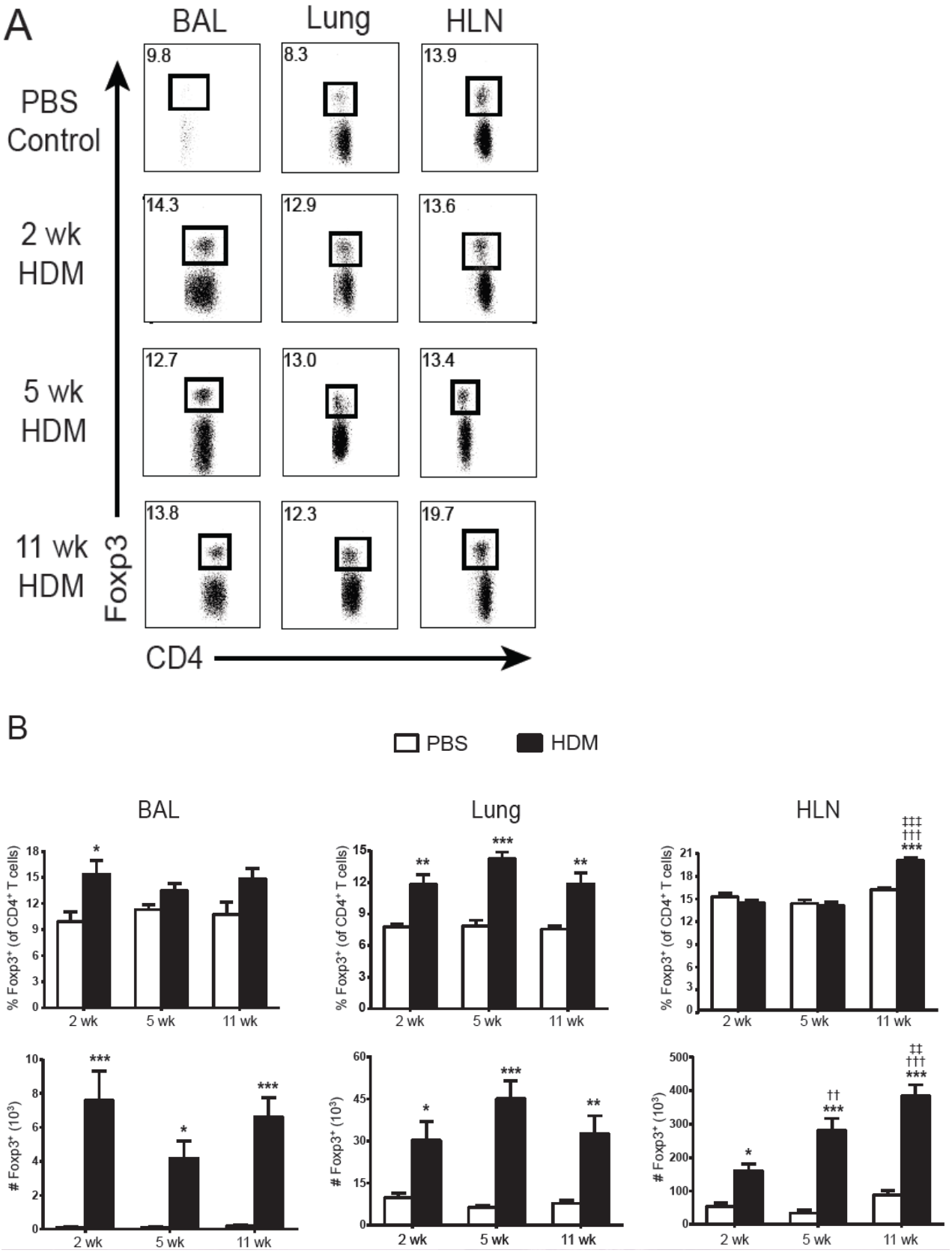
A
BAL
Lung
HLN
PBS Control
2 wk HDM
5 wk HDM
11 wk HDM
9.8
8.3
13.9
14.3
12.9
13.6
12.7
13.0
13.4
13.8
12.3
19.7
Foxp3
CD4
B
PBS
HDM
BAL
Lung
HLN
% Foxp3+ (of CD4+ T cells)
# Foxp3+ (10³)
2 wk
5 wk
11 wk

Figure 13. Foxp3^{+} Treg numbers increase steadily in the HLN over the course of HDM exposure and correlate with suppression of AAD. **(A)** Representative flow cytometry dot plots from BAL, lung tissue, and HLN of PBS control (short-term) and HDM-exposed C57BL/6 females. Cells were gated on CD3^{+}CD4^{+} T lymphocytes and Foxp3 expression was examined. **(B)** Total percentages and numbers of Foxp3^{+} Tregs of all CD4+ T cells in PBS control or HDM mice. Data represent mean ± SEM values; n = 10-13 per group (PBS control; values pooled from 3 independent experiments), 15-22 per group (HDM; values pooled from 3-5 independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. time-matched PBS control; †††$p < 0.001$ vs. short-term HDM; ‡‡$p<0.01$, ‡‡‡ $p < 0.001$ vs. intermediate-term HDM.

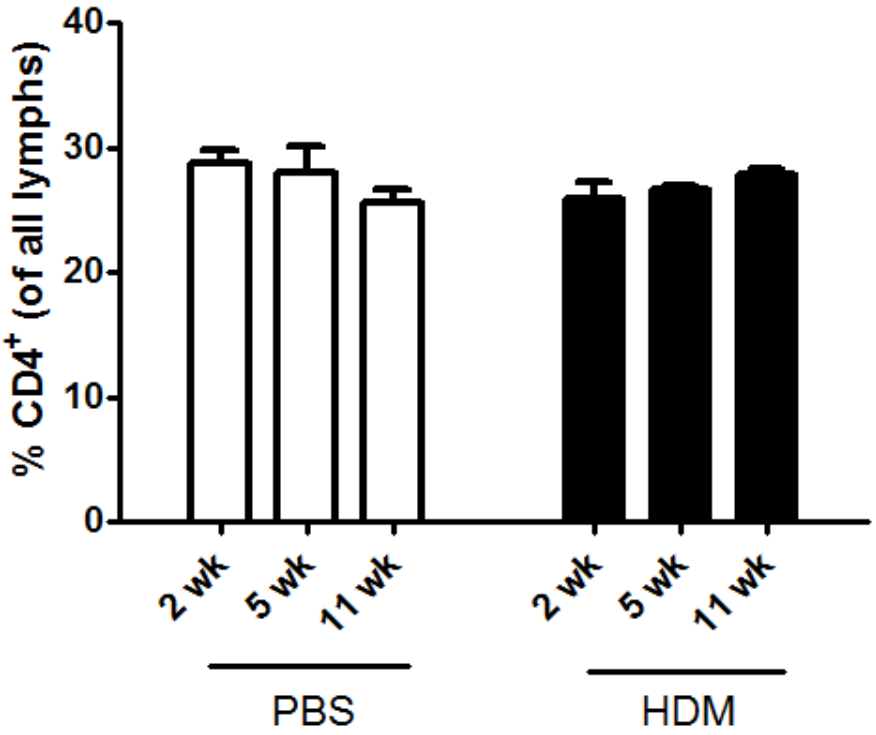

Figure 14. CD4+ T lymphocyte frequency in the HLN does not differ across the various stages of the model. Total percentage of $CD3^{+}CD4^{+}$ T lymphocytes were calculated as a frequency of total, live lymphocytes (gated on the basis of forward and side scatter) in PBS control and HDM mice. Data represent mean ± SEM values; n = 10-13 per group (PBS control; values pooled from 3 independent experiments), 15-22 per group (HDM; values pooled from 3-5 independent experiments).

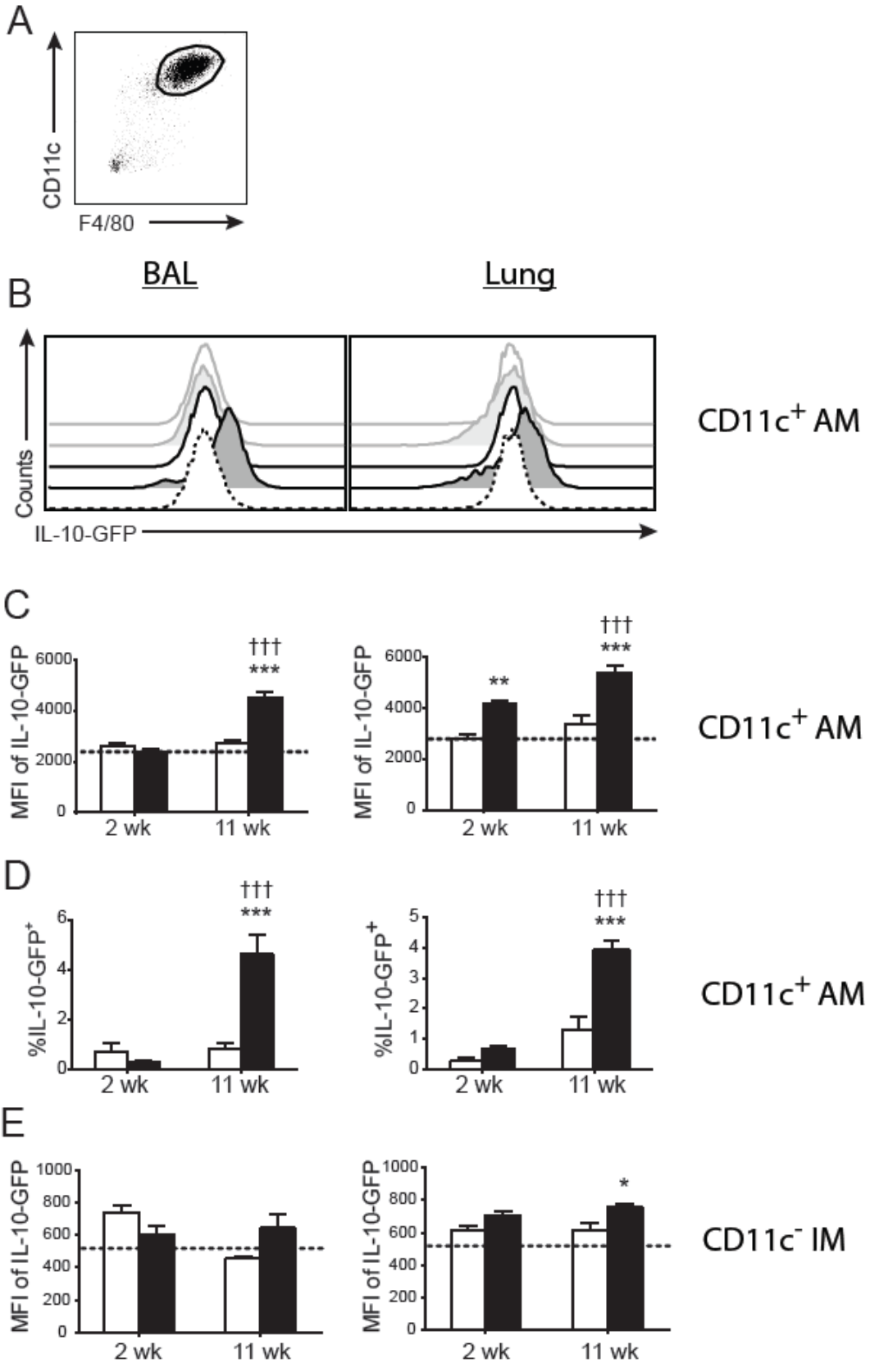
A
CD11c
F4/80
BAL
Lung
B
Counts
IL-10-GFP
CD11c+ AM
C
MFI of IL-10-GFP
6000
4000
2000
0
2 wk
11 wk
†††
***
**
CD11c+ AM
D
%IL-10-GFP+
6
4
2
0
5
3
1
CD11c+ AM
E
1000
800
600
400
200
*
CD11c- IM

Figure 15. AMs assume an IL-10^{+} phenotype in the BAL and lung tissue following long-term HDM exposure. Male and female Vert-x mice were subjected to short-term or long-term PBS or HDM exposure as shown in Chapter 2, *Fig 2*. **(A)** Sample FACS plot demonstrating gating for F4/80^{+}CD11c^{+} AMs (encircled) among the population of total BAL leukocytes in short-term PBS control mouse. **(B)** Representative histogram plot of IL-10-GFP expression in CD11c^{+} AMs. Solid, light grey-lined histograms represent short-term Ag exposure in PBS control (unshaded) or HDM (shaded) mice. Solid, dark grey-lined histograms represent long-term Ag exposure in PBS control (unshaded) or HDM (shaded) mice. Dotted histogram represents negative control (GFP^{-} AMs from a WT mouse). **(C)** MFI of IL-10-GFP expression in CD11c^{+} AMs from PBS control (unshaded bars) or HDM (shaded bars) mice. Dotted line represents negative control (GFP^{-} AMs from a WT mouse). **(D)** Frequency of IL-10-GFP^{+} macrophages expressed as % total AMs from PBS control (unshaded bars) and HDM (shaded bars) mice. **(E)** MFI of IL-10-GFP expression in CD11c^{-} IMs from PBS control (unshaded bars) or HDM (shaded bars) mice. Dotted line represents MFI of GFP^{-} IMs from a WT mouse. Data represent mean ± SEM values (combined for male and females); n = 5-6 per group (representative of three independent experiments). *$p < 0.05$, ***$p < 0.001$ vs. time-matched PBS control; ††† vs. short-term HDM.

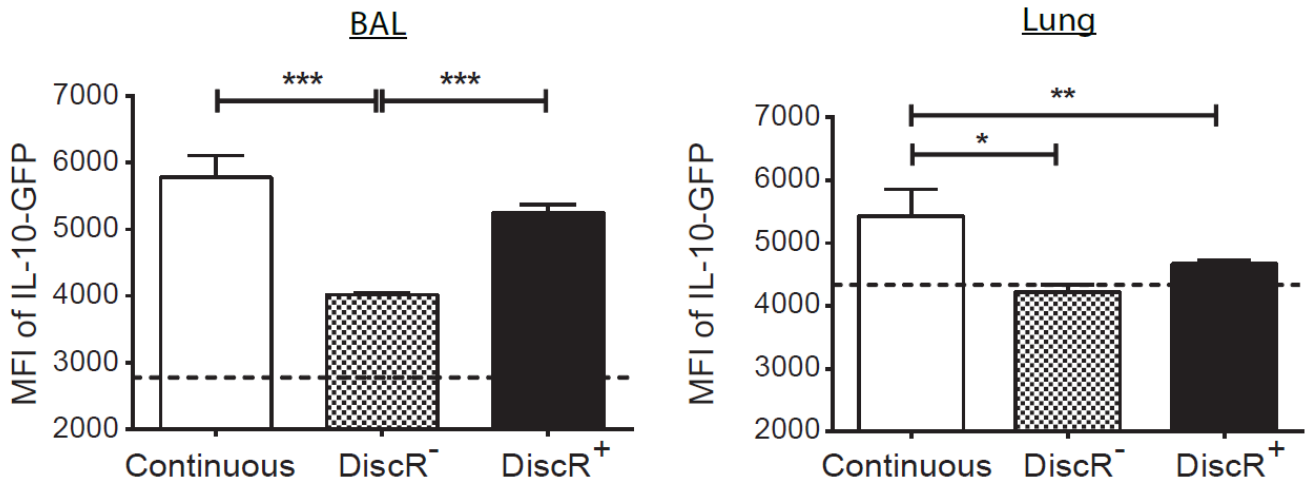

Figure 16. AMs in the BAL of mice discontinuously exposed to HDM produce IL-10. MFI of IL-10-GFP expression in $CD11c^+$ AMs from 11 wk continuous, $DiscR^-$, and $DiscR^+$ mice. Dotted line represents MFI of GFP^- AMs from a WT mouse. Data represent mean ± SEM values; n = 5-6 per group, *p < 0.05, **p < 0.01, ***p < 0.001.

CHAPTER 4

THE ROLE OF IL-10 AND ALVEOLAR MACROPHAGES IN TOLERANCE DEVELOPMENT AGAINST HOUSE DUST MITE

Introduction

The respiratory tract is continuously subjected to a variety of innocuous Ags[197]. Although this exposure commonly results in the development of immunologic tolerance or non-responsiveness against these Ags, lack of tolerance generation can lead to allergic asthma[158,198,199]. Despite the magnitude of this problem, many pharmacologic therapies for asthma are primarily geared towards symptom relief and exert few, if any, effects on long-term disease progression[200]. It has become critical to dissect the mechanisms that affect tolerance development in order to target improved therapies that can restore normal immunity and quality of life in asthmatic patients.

Regulatory T cells (Tregs) are essential for suppressing immune responses against non-pathogenic Ags[186]. Due to the fact that Tregs are often decreased in asthmatic airways, methods to expand Tregs and enhance their suppressive potential has been the focus of many recent studies[59,60]. However, the mechanism by which Tregs are generated in the process of tolerance formation against inhaled allergens has not been well established.

AMs play a key role in the defense against inhaled pathogens and allergens and can transition between a number of functional phenotypes exhibiting opposing roles (i.e. pro-inflammatory versus anti-inflammatory, immunogenic versus tolerogenic)[75]. Previous findings in our long-term HDM model have

shown that suppression of AAD is associated with an accumulation of IL-10$^+$ AMs in the BAL and lungs of HDM-tolerant mice. These cells (traditionally thought of as "regulatory" AMs) primarily arise during late stages of the immune response and can downregulate inflammation[201] through production of anti-inflammatory cytokines[202,203]. Although the exact mechanism by which IL-10$^+$ AMs exert their regulatory activities has not been described, one possibility is that they induce Foxp3$^+$ Tregs through their secretion of IL-10 and TGF-β, a theory that has been supported by human and mouse studies alike[103,204-206]. Moreover, we have observed concurrent increases in both Foxp3$^+$ Tregs and IL-10$^+$ AMs at the site of inflammation in HDM-tolerant mice (Chapter 3, *Figs 13 and 15*). However, while the contributions of Foxp3$^+$ Tregs in the suppression of asthmatic responses have been long accepted, the role of IL-10$^+$ AMs in tolerance against inhaled allergens has not been well-documented.

The purpose of this study was to evaluate the contribution of IL-10$^+$ AMs to the induction of immunologic tolerance against long-term HDM exposure. Our results demonstrated that neither IL-10 nor IL-10$^+$ AMs are likely to be the essential contributors to the suppression of AAD seen with long-term HDM exposure in mice with otherwise intact immune systems. However, AMs from long-term HDM mice showed an enhanced ability to induce Foxp3$^+$ Tregs *in vitro*, suggesting that these cells do have regulatory functions and can contribute to the overall attenuation of AAD observed in this long-term HDM model.

Results

IL-10 was nonessential for the suppression of allergic inflammation with long-term HDM exposure

To determine whether IL-10 was required for the development of tolerance to HDM, total leukocyte counts and cellular differentials were performed in wild type (WT) and IL-10$^{-/-}$ mice following short-term or long-term HDM instillation. No differences were seen in total BAL, HLN, or lung leukocyte counts prior to the start of HDM exposure (*Fig 17A*). Following short-term HDM exposure, IL-10$^{-/-}$ mice trended towards increased leukocyte counts in all three lung compartments relative to WT mice, though

these differences were not statistically significant. However, following long-term HDM exposure, WT and IL-10$^{-/-}$ mice demonstrated similar leukocyte counts in the BAL, HLN, and lungs.

As expected, neither WT nor IL-10$^{-/-}$ mice had any evidence of BAL eosinophilia prior to HDM exposure (*Fig 17B*). Following short-term HDM exposure, eosinophil levels increased to ~40-45% in both sets of mice, but declined to <3% following long-term HDM exposure. Additionally, total IgE levels, which were non-significantly elevated in IL-10$^{-/-}$ mice relative to WT mice following short-term HDM exposure, declined significantly following long-term HDM exposure (*Fig 17C*). Together, these data suggest that IL-10 is dispensable for tolerance generation and subsequent suppression of allergic inflammation following long-term HDM exposure.

IL-10$^{-/-}$ animals did not demonstrate AHR with intermediate-term HDM exposure

Although airway inflammation may contribute to the development of airflow obstruction, it does not always correlate well with pulmonary function[207]. To determine whether IL-10 was required for suppression of AHR with long-term HDM exposure despite its seemingly dispensable role in suppression of allergic inflammation, WT and IL-10$^{-/-}$ mice were administered HDM for short-, intermediate-, and long-term periods prior to methacholine challenge. No differences were observed between methacholine challenge curves of WT and IL-10$^{-/-}$ mice in their naïve states or after short-term HDM challenge (*Fig 18*). However, while WT mice showed peak AHR following intermediate-term HDM exposure, IL-10$^{-/-}$ demonstrated a significantly decreased response with no evidence of AHR even at higher doses of methacholine. Furthermore, while long-term HDM exposure was associated with resolution of AHR responses in WT mice, IL-10$^{-/-}$ mice simply maintained their relative lack of AHR. Together, these data suggest that while WT mice are capable of exhibiting a biphasic AHR response in response to HDM instillation, IL-10$^{-/-}$ mice do not demonstrate any evidence of AHR with intermediate-term HDM exposure.

IL-10$^{-/-}$ animals had a lower frequency of local Foxp3^{+} Tregs upon allergen challenge than WT animals

Since IL-10$^{-/-}$ mice appeared to be capable of inducing immunologic tolerance to long-term HDM administration in a similar manner as WT mice (as demonstrated by suppression of BAL eosinophilia), we examined percentages of Foxp3^{+} Tregs in the lung compartments of IL-10$^{-/-}$ mice to determine whether similar increases in Treg levels were noted at tolerance as were seen in WT HLNs (Chapter 3, *Fig 13*). While no differences were observed in Treg frequency in the BAL, HLN, or lung tissue of naïve WT and IL-10$^{-/-}$ animals (*data not shown*), short-term HDM-exposed IL-10$^{-/-}$ mice demonstrated significantly decreased Treg levels in the BAL and HLN when compared to WT animals (*Fig 19*). Moreover, this decrease in Treg frequency was found to be significant in all three lung compartments following long-term HDM exposure. Interestingly, while BAL and lung tissue Treg percentages were further reduced in long-term HDM-exposed IL-10$^{-/-}$ mice relative to short-term HDM-exposed IL-10$^{-/-}$ mice, HLN Tregs exhibited the opposite trend, a finding consistent with what we have previously observed in WT animals. These data support the idea that the HLN is the most critical site for Treg accumulation and subsequent suppression of AAD responses.

AMs from long-term HDM mice induced Foxp3^{+} Tregs in vitro

Despite suppression of BAL eosinophilia in IL-10$^{-/-}$ mice with long-term HDM exposure, macrophages did not appear to have the same gross morphological features (i.e. were neither enlarged when compared to naïve or short-term HDM-exposed macrophages nor multinucleated) like those seen in WT mice following long-term HDM exposure (Chapter 3, *Fig 6D, Figs 20*A-C). These findings supported the idea that the enlarged, multinucleated macrophages seen in long-term HDM-exposed WT mice are IL-10^{+} cells. Since IL-10$^{-/-}$ mice also demonstrated decreased Treg levels when compared to WT mice, we hypothesized that IL-10^{+} "regulatory" AMs may contribute to Treg induction. To test this hypothesis, we performed Treg induction assays using AMs from short-term HDM-exposed mice, long-term HDM-exposed mice, and time-matched control (11 wks PBS) mice. Foxp3^{+} Treg frequency in the

purified naïve Teff population prior to co-culture with AMs was <2%. In the absence of exogenous TGF-β, AMs did not induce Tregs in any of the groups, suggesting that AMs do not produce significant amounts of TGF-β at any stage of the model (*Fig 21A*). In the presence of exogenous TGF-β, AMs from short-term HDM mice and long-term PBS mice induced ~16-17% Tregs, a frequency that doubled when AMs from lungs of long-term HDM mice were used as the APC. Together, these findings suggested that long-term HDM-exposed AMs have an enhanced ability to induce $Foxp3^+$ Tregs relative to AMs from short-term HDM mice or time-matched PBS control mice.

To determine whether the increase in Treg induction seen with long-term HDM-exposed AMs was the result of their enhanced IL-10 production, similar co-culture experiments were performed with αCD3 and TGF-β in the presence or absence of neutralizing antibodies to IL-10 and IL-10R. AMs from long-term HDM mice induced 10% more Tregs than AMs from short-term HDM mice and 5% more Tregs than AMs from long-term PBS control mice in the presence of αCD3 and exogenous TGF-β; however, this effect was not significantly inhibited by blockade of IL-10 and IL-10R signaling (*Fig 21B*). Low-level expression of MHC II and co-stimulatory markers on APCs has been thought to enhance their Treg- inducing abilities. When compared to PBS control animals, AMs in HDM-exposed animals demonstrated a significant upregulation in MHC II expression (*Fig 21C*). However, long-term HDM exposure resulted in a significant decline in MHC II levels on AMs relative to short-term HDM exposure, further potentiating their regulatory capacity.

Depletion of AMs did not affect tolerance development with long-term HDM exposure

To next determine whether AMs from long-term HDM-exposed mice have an *in vivo* role in tolerance induction, we depleted AMs in long-term HDM-exposed mice using intranasal injections of Cl_2MDP (clodronate) liposomes. Studies in Vert-x mice have shown that IL-10^+ AMs first appear in the BAL and lungs after intermediate-term (5 wks) HDM exposure, as was demonstrated by a rightward shift in the GFP expression curve (*Fig 22A*) representing significantly increased MFI of IL-10 in $CD11c^+$ AMs relative to PBS control animals (*Fig 22B*). Intermediate-term HDM-exposed Vert-x mice also showed a

significantly increased percentage of IL-10$^+$ AMs relative to control animals (*Fig 22C*) that appeared before the period when eosinophilia has been shown to resolve (~7-8 weeks). Thus, the AM depletion protocol shown in Chapter 2, *Fig 4* was devised to (more specifically) deplete IL-10$^+$ AMs.

Weekly, intranasal administration of Cl_2MDP to either i.n. PBS- or HDM-exposed resulted in 80-85% depletion of AMs in both the BAL and the lungs (*Fig 23A*). To next test the hypothesis that AM depletion would prevent suppression of allergic inflammation with long-term HDM exposure, we attempted to determine eosinophil frequencies in the airways of Cl_2MDP-treated mice but were unable to manually perform cellular counts and differentials due to widespread AM death. However, FACS analysis demonstrated that variable percentages of Siglec-F$^+$CD11b$^+$CD11c$^-$ eosinophils were present in the BAL of HDM-exposed, Cl_2MDP-treated animals but not animals administered control liposomes (*data not shown*). This suggested that depletion of AMs after intermediate-term HDM exposure may have inhibited generation of tolerance to long-term HDM; however, this could not be reliably confirmed through assessment of inflammatory parameters due to the limitations described above.

To better test the hypothesis that AMs are involved in generation of tolerance to HDM, Cl_2MDP- or control-treated mice that were exposed to long-term, i.n. PBS or HDM were subjected to methacholine challenge to determine whether AM depletion inhibited suppression of AHR responses in HDM-instilled animals. As expected, long-term PBS and HDM mice administered control liposomes showed no evidence of AHR (*Fig 23B*). Interestingly, Cl_2MDP treatment did not alter AHR responses in either PBS control or HDM mice, suggesting that AMs do not play a critical role in suppression of AAD with long-term HDM exposure.

In vivo AM depletion did not affect Foxp3$^+$ Tregs in the HLN

In order to examine the question of whether IL-10$^+$ AMs influence Foxp3$^+$ Treg induction *in vivo*, Treg frequencies were examined in the HLNs of long-term HDM-exposed mice after Cl_2MDP liposome administration. PBS control mice had similar levels of Tregs in the HLN regardless of whether they were given Cl_2MDP or control liposomes (*Fig 24A*). HDM mice demonstrated comparable levels of HLN

Tregs as PBS control animals regardless of liposome treatment type, indicating that AMs do not much influence accumulation of Tregs in the HLN in long-term HDM mice.

Because AMs are not thought to migrate to the HLN in significant numbers, we wanted to determine whether regulatory interactions can occur between AMs and $CD4^+$ T cells in the lung. Confocal imaging of lung sections from long-term HDM-exposed mice revealed clusters of inflammatory cells surrounding $Epcam^+$ airway epithelial cells (*Fig 24B*) that had previously been appreciated on H&E staining (Chapter 3, *Fig 9D*). These clusters were primarily comprised of $F4/80^+CD11c^-$ IMs and $CD4^+$ T cells, which were found to be in close proximity to one another. On the other hand, $F4/80^+CD11c^+$ AMs (purple) were located diffusely throughout the alveoli and showed minimal interactions with $CD4^+$ T cell clusters in the lung. Together, these data suggest that AMs are not likely to influence Treg induction in the lung tissue in a contact-dependent manner.

Discussion

Alveolar macrophages (AM) have been termed "the forgotten cell in asthma"[208] because despite their importance for maintaining homeostasis in the lung, their anti-inflammatory functions have not been extensively characterized. The present findings demonstrate that $IL\text{-}10^+$AMs, which were generated following intermediate-term HDM exposure and maintained in the BAL and lungs of HDM-tolerant animals (Chapter 3), had regulatory properties and were capable of inducing $Foxp3^+$ Tregs *in vitro*. However, these macrophages appeared to have a non-essential role in tolerance generation against HDM *in vivo*.

IL-10 is well known for its ability to dampen excessive inflammatory responses and is thought to play a role in the maintenance of tolerance against inhaled allergens. However, its critical function in the induction of tolerance has, to the best of our knowledge, not definitely been demonstrated. When WT and $IL\text{-}10^{-/-}$ animals were administered i.n. HDM, both developed AAD at similar severities following short-term allergen exposure, as demonstrated by comparable leukocyte counts, eosinophil levels, and non-significant differences in total IgE levels. However, with long-term HDM exposure, $IL\text{-}10^{-/-}$ mice were

capable of developing immune tolerance to the same degree if not better than WT mice, as was demonstrated by their significantly reduced total serum IgE levels. These data are in agreement with previous findings from our laboratory, which have shown that IL-10 is non-essential for the formation of tolerance in our OVA model[209]. Although suppression of airway inflammation in our HDM model was not affected by loss of IL-10, the influence of IL-10 on resolution of AHR could not be evaluated since IL-10-deficient mice did not develop AHR upon intermediate-term HDM exposure. Other groups have also demonstrated lack of AHR upon methacholine challenge in IL-$10^{-/-}$ animals despite the fact that Th2 inflammatory responses are not inhibited[194,210,211]. Moreover, this protective phenotype can be 1) disrupted if IL-10 is reconstituted in IL-$10^{-/-}$ mice and 2) transferred if splenocytes from allergen-sensitized IL-$10^{-/-}$ mice are injected into sensitized SCID mice prior to allergen challenge[211]. Although the mechanism behind this protection in not entirely understood, it has been suggested that IL-$10^{-/-}$ mice have increased IL-13Rα2, a decoy receptor for IL-13[194]. Collaboration between these two molecules is likely responsible for the phenotype observed in IL-$10^{-/-}$ mice following allergen sensitization and challenge, with IL-10 functioning as the dominant inhibitor of Th2 inflammation and IL-13Rα2 as a key inhibitor of AHR and mucus hypersecretion.

Given the aforementioned results, it would seem likely that IL-10 acts as a double-edged sword in the suppression of AAD seen with long-term HDM exposure. While its suppressive effects on allergic airway inflammation are well-documented, the mechanism by which IL-10 facilitates development of AHR is not known. However, it must act in concert with one or more inflammatory factors to increase smooth muscle reactivity because AHR has not been observed naïve, IL-10-competent animals. In addition, administration of IL-10 alone in WT mice has not been shown to augment AHR[212]. Thus, the biphasic AHR response observed in intermediate-term HDM mice and long-term HDM mice despite the presence of IL-10^{+} AMs in the lungs of both groups is likely to hinge on the absence of a key inflammatory mediator in the latter set. Follow-up studies should be done to identify this factor, although

we have shown that IL-13 does not decrease with long-term HDM exposure and that IL-17 is not detectable across any stage of the model (*data not shown*).

Despite suppression of allergic inflammation in IL-$10^{-/-}$ mice with long-term HDM exposure, "regulatory" AMs were not thought to develop in these animals, as they did not bear any gross resemblance to the macrophages observed in the BAL of long-term HDM-exposed WT mice. Unfortunately, we were unable to test this hypothesis by FACS analysis since IL-10 was used as the defining marker of these anti-inflammatory cells. Thus, additional studies should be performed to better characterize these macrophages in WT mice so that other reliable phenotypic markers of these AMs can be identified. Yet what was most intriguing was that although long-term HDM-exposed WT and IL-$10^{-/-}$ mice both displayed similar tolerogenic phenotypes, $Foxp3^+$ Tregs were reduced in the BAL, lung, and HLN of IL-$10^{-/-}$ mice relative to their WT counterparts, suggesting a critical role for IL-10 in the induction and/or maintenance of $Foxp3^+$ Tregs. Both functions of IL-10 have been previously demonstrated by other groups[104,105]; however, Denning et al. were among the few who have shown an essential role for IL-10/IL-10R signaling in the Ag-specific generation of $Foxp3^+$ Tregs. In fact, one of the most interesting observations made by the Pulendran laboratory was that lamina propria macrophages were the primary source of the IL-10 required for Treg generation[104] and were more efficient in inducing Tregs than lamina propria DCs in the presence of exogenous TGF-β. Naïve AMs and lung-resident macrophages are other examples of mucosal M2 macrophages capable of inducing $Foxp3^+$ Tregs and do so in a TGF-β- and retinoic acid-dependent but IL-10-independent manner [98,103]. In contrast with the work by Coleman et al. and Soroosh et al., we did not find that our AMs were able to produce significant amounts of TGF-β, suggesting that these cells transition to a different subtype of macrophage (either M1 or TGF-β^- M2 cells) once they are exposed to allergens. However, it was clearly evident that in the presence of exogenous TGF-β, IL-10^+ AMs from the lungs of long-term HDM mice were capable of inducing a greater percentage of $Foxp3^+$ Tregs than IL-10^- AMs from short-term HDM mice. This is likely explained by the fact that short-term HDM exposure upregulates macrophage production of M1 cytokines (IL-1, IL-6, TNFα)[103], MHC II, and co-stimulatory markers, thus inhibiting their ability to

effectively generate Treg cells. We have shown that with long-term HDM exposure, AMs regain their ability to generate Tregs, perhaps by altering their surface marker and cytokine profile to a more anti-inflammatory nature. Along these lines, the fact that AMs from long-term HDM mice did not consistently induce more Tregs than long-term PBS-exposed AMs can be attributed to the low activation status (e.g. low MHC II levels) of the latter population.

Unlike the study conducted by Denning et al., IL-10/IL-10R signaling did not appear to have a significant role in AM-mediated induction of Tregs. However, inhibiting this signaling pathway was associated with trending decreases in Treg induction in all three experimental groups. A more significant inhibition on Treg induction may have been noted if a greater concentration of anti-IL-10 and anti-IL-10R antibody had been used; in fact, the results from Denning et al. suggest that at least twice as much anti-IL-10 and anti-IL-10R are recommended[104]. Thus, our studies should be repeated with larger doses of neutralizing antibodies. However, other potential targets should also be considered when accounting for the ability of long-term HDM AMs to induce more Tregs than AMs from short-term HDM mice, including retinoic acid (*see Chapter 6, Future Directions*).

In order to study the *in vivo* relevance of AMs in tolerance formation against HDM, clodronate liposomes were administered i.n. to long-term HDM-exposed mice, which depleted over 80% of AMs in the BAL and lungs. While we lacked the ability to specifically deplete IL-10^+ macrophages within these studies, we attempted to work around this issue by administering clodronate liposomes specifically during the critical period when these cells appear and tolerance begins to form (weeks 5-9). Depletion of AMs during this critical period did not prevent the suppression of AHR associated with long-term HDM exposure, suggesting that AMs are not essential for development of immunologic tolerance to HDM. This contrasts with previous studies, which have shown that AMs from allergy-resistant animals protect against the development of AHR[92]. However, this may be explained by the fact that we likely depleted both pro- and anti-inflammatory AM populations with clodronate liposome administration during weeks 5-9. Thus, it is difficult to determine whether the depletion of pro- and anti-inflammatory AMs negated the protective effects of IL-10^+ AMs on AHR or whether AMs truly lack a protective role in this model.

Long-term HDM exposure and subsequent development of tolerance is associated with increases in regulatory lymphocytes within the HLN (Chapter 3), which we have previously shown can attenuate severity of AAD when adoptively transferred into $RAG^{-/-}$ mice prior to allergen challenge[150]. Although AMs were capable of inducing Tregs *in vitro*, depletion of AMs did not reduce Treg frequency in the HLN, demonstrating that these cells may not impact induction of Tregs within this critical site of immune regulation. Although AMs do have the capacity to migrate to the HLN[213], studies have shown that transfer of Ag-pulsed lung macrophages to lymphotoxin β receptor-deficient mice that do not possess peripheral lymph nodes does not inhibit the ability of these cells to induce iTregs[103]. This supports our finding that AMs do not have an obligate role in promoting HLN Treg generation. However, the one caveat in these data was the fact that long-term HDM mice administered control liposomes did not demonstrate increased Tregs over time-matched PBS control mice as was expected based on results described in Chapter 3. However, the fact that these HDM-instilled, control liposome-treated mice demonstrated suppression of AHR responses despite lack of Treg increases in the HLN raises the additional question of how critical Tregs truly are to this overall process.

Ag-pulsed lung macrophages transferred intratracheally into naïve, immune-competent mice in conjunction with naïve $Foxp3^-$ Ag-specific T cells have been shown to convert a significant percentage of these T cells to $Foxp3^+$ iTregs within the lungs[103]. We have previously demonstrated that $Foxp3^+$ Tregs increase in the lungs of HDM-exposed animals relative to PBS controls at all time points, suggesting that HDM exposure leads to Treg induction in the lungs. However, no specific increases in Tregs were previously observed in the lungs of long-term HDM mice relative to short-term or intermediate-term HDM mice. If Tregs are indeed generated at a greater frequency by HDM-tolerant AMs *in vivo*, it is possible that the newly induced cells may migrate immediately to the HLN, where they slowly accumulate over time to eventually dampen Th2 responses (Chapter 3, *Fig 13*). Nevertheless, it is important to note that if this phenomenon does indeed occur, AMs likely play an ancillary role in this process, as they were unable to produce TGF-β and demonstrated minimal cell-cell contact with T cells in the lung.

In conclusion, the present study demonstrated a non-essential role for IL-10 and IL-10$^+$ AMs in tolerance generation against long-term HDM. Nevertheless, IL-10$^+$ AMs from HDM-tolerant mice were shown to have an enhanced capacity to convert naïve Teff to Foxp3$^+$ iTregs *in vitro*, the first data of its kind to demonstrate that allergen-exposed AMs regain their ability to induce Tregs if Ag exposure persists for a chronic and continuous period of time. These data imply that AMs from long-term HDM mice are truly regulatory cells; however, their contributory effects may be masked by a plethora of additional regulatory mechanisms that can compensate for their absence *in vivo*. This redundancy in compensatory mechanisms may explain why the vast majority of people do not develop allergic responses upon exposure to inhaled allergens. A combination of regulatory deficits (including lack of IL-10$^+$ AM development) may in fact be required to induce an asthmatic response. Thus, additional studies on the utility of IL-10$^+$ AMs to skew the immune system towards a regulatory response are certainly warranted.

Figures

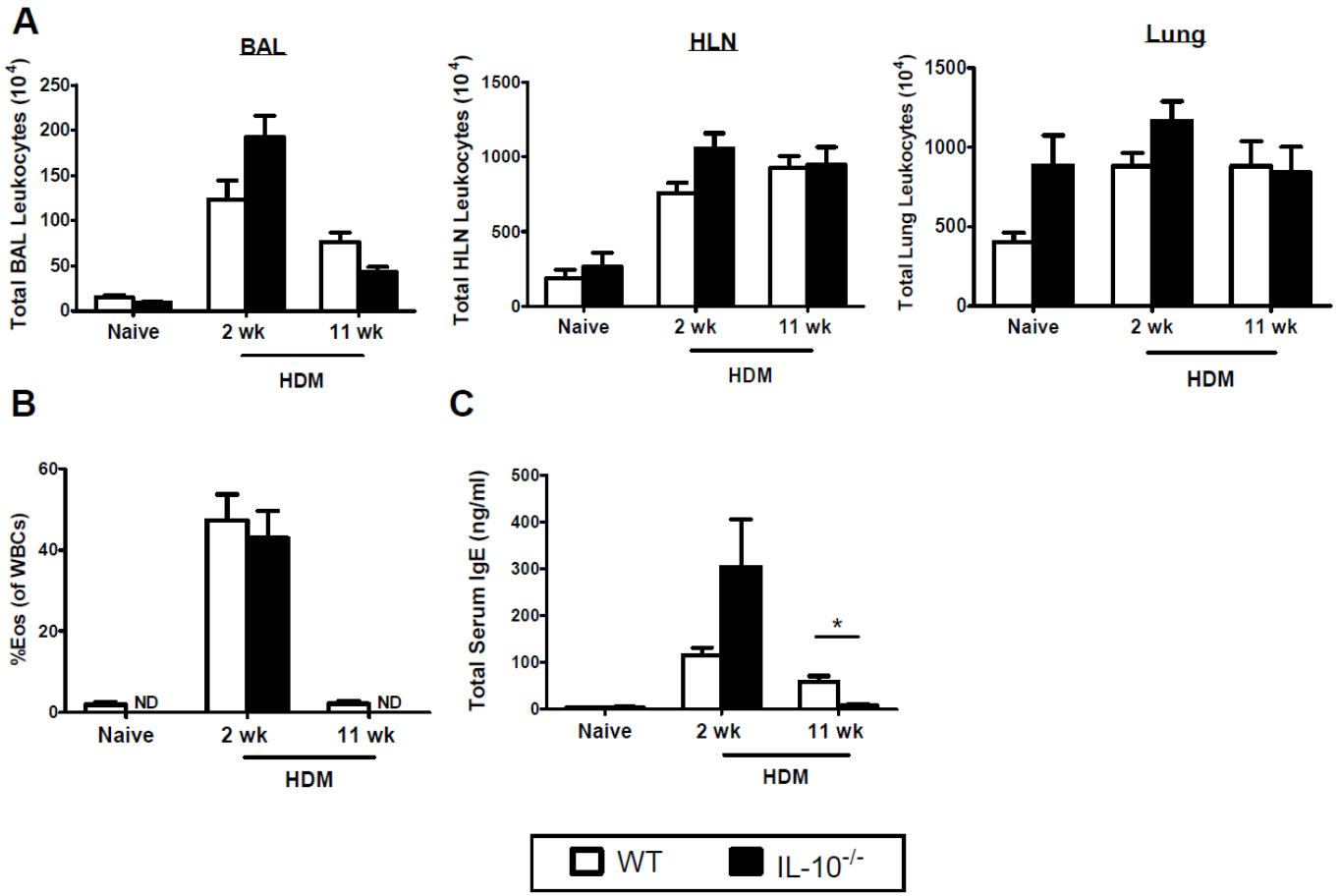

Figure 17. IL-10 is not critical for suppression of eosinophilic inflammation in response to long-term HDM exposure. Female IL-$10^{-/-}$ or WT (C57BL/6) mice were exposed i.n. HDM extract for up to 2 or 11 wks as described in Chapter 2, *Fig 2*. **(A)** At sacrifice, leukocytes from BAL, HLN, and lungs were harvested and manually counted on a hemocytometer. **(B)** Cytocentrifuged BAL cell preparations were stained with May-Grünwald Giemsa and differential analysis was manually performed. **(C)** Total serum IgE levels were determined via ELISA. Statistical comparisons were made between WT and IL-$10^{-/-}$ mice. *$p<0.05$

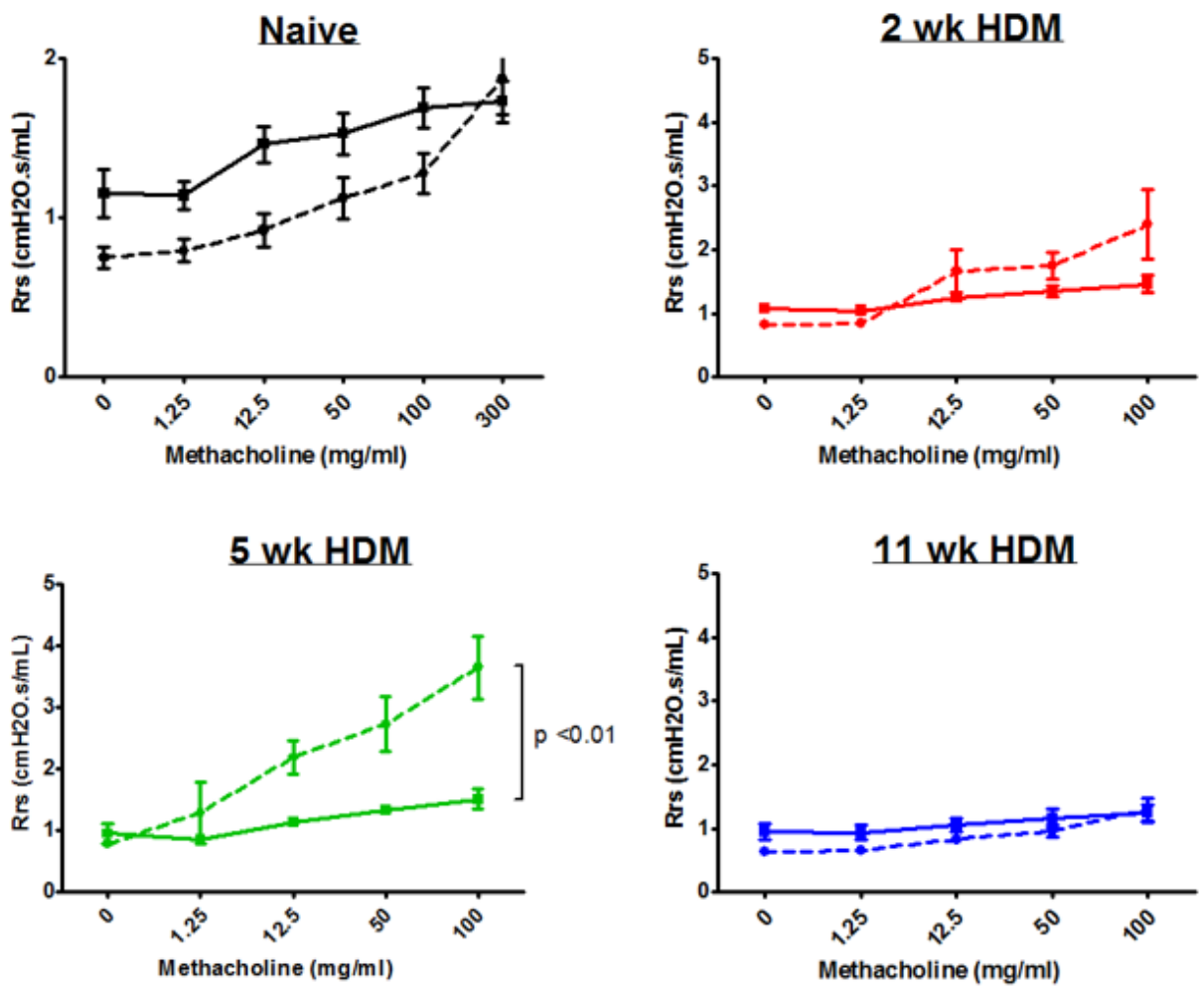

Figure 18. IL-10$^{-/-}$ mice do not exhibit AHR in response to intermediate-term HDM exposure. Female WT (dotted lines) or IL-10$^{-/-}$ (solid lines) mice underwent tracheostomy and were mechanically ventilated prior to methacholine exposure. Changes in Rrs responses to increasing doses of aerosolized methacholine were determined using the flexiVent system (SCIREQ). Data represent mean ± SEM values; n =5 per group. For statistical purposes, group comparisons were based on area under the curve (AUC) measurements.

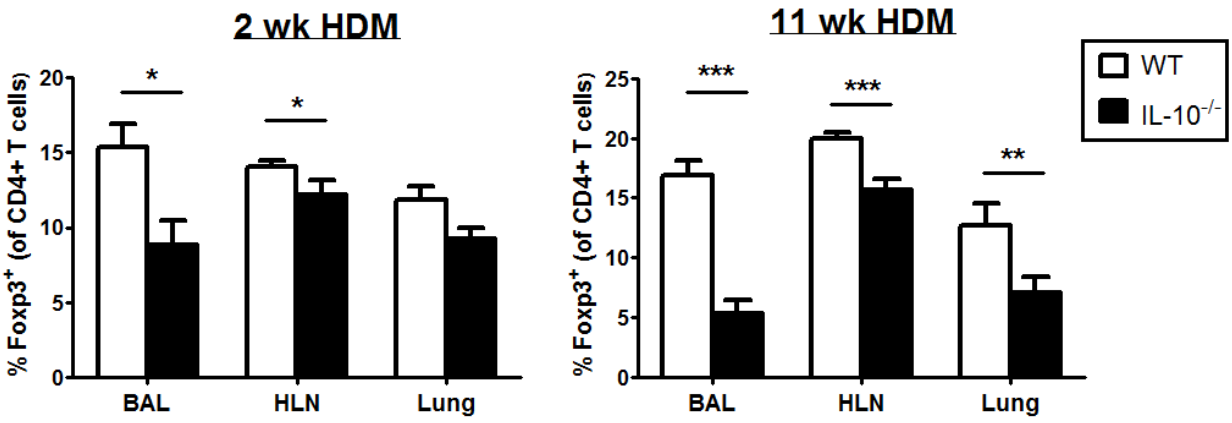

Figure 19. Foxp3^{+} Treg numbers are significantly decreased in the lung compartments of HDM-exposed IL-10$^{-/-}$ mice relative to WT mice. Total percentages of Foxp3^{+} Tregs of all CD4+ T cells in WT and IL-10$^{-/-}$ mice subjected to either 2 or 11 wks of i.n. HDM. Data represent mean ± SEM values; n = 4-5 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

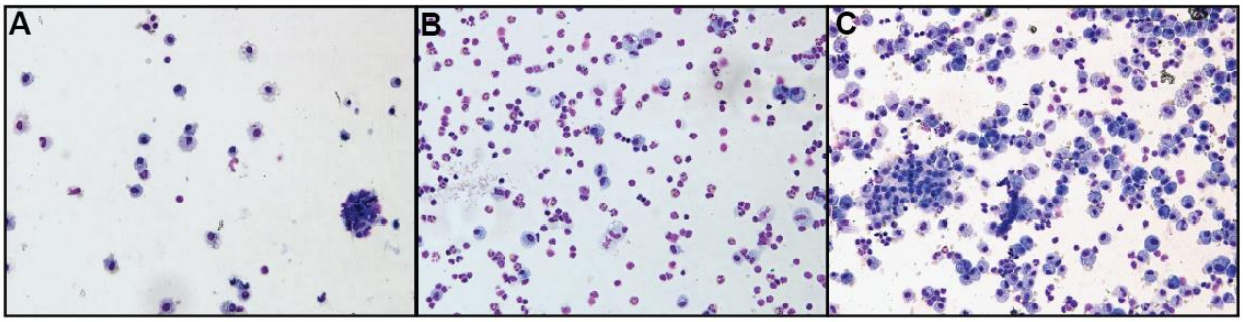

Figure 20. Macrophages do not become enlarged and multinucleated in IL-10$^{-/-}$ mice administered long-term HDM. **(A)** Naïve, female IL-10$^{-/-}$ mice were exposed to i.n. HDM extract for either **(B)** 2 wks or **(C)** 11 wks as described in Chapter 2, *Fig 2*. At sacrifice, total BAL leukocytes were harvested and cytocentrifuged preparations were stained with May-Grünwald Giemsa. 20X magnification.

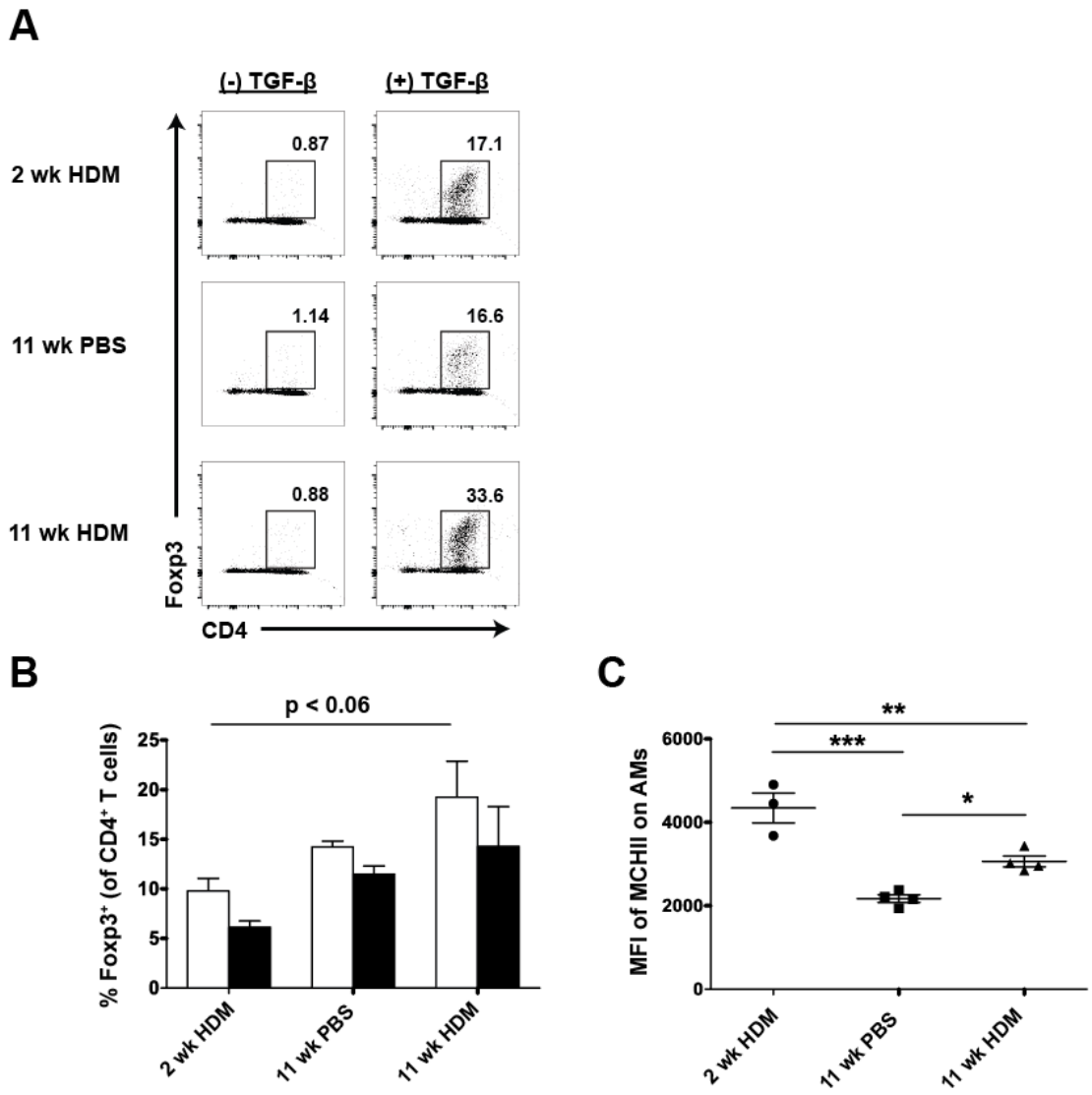

Figure 21. AMs from long-term HDM-exposed mice induce Foxp3^{+} Tregs in the presence of exogenous TGF-β. **(A)** AMs isolated from the lungs (6-7 lungs pooled per group) of short-term HDM, long-term PBS, or long-term HDM were isolated at a 1:1 ratio with naïve Teff (isolated from the spleens of naïve Foxp3^{gfp} mice) in the presence of αCD3 ± TGF-β for 72 hours. Plots represent frequency of GFP-expressing (Foxp3^{+}) Tregs of all Teff. **(B)** Similar co-cultures were repeated in the presence of αCD3 and TGF-β ± αIL-10 and αIL-10R neutralizing antibodies (2 lungs pooled per sample, n = 3-4 samples per group). Unshaded bars represent samples without αIL-10/IL-10R blockade and shaded bars represent samples with αIL-10 and αIL-10R blockade. **(C)** MFI of MHCII on sorted AMs from (B) prior to co-culture. *p<0.05, **p<0.01, ***p<0.001

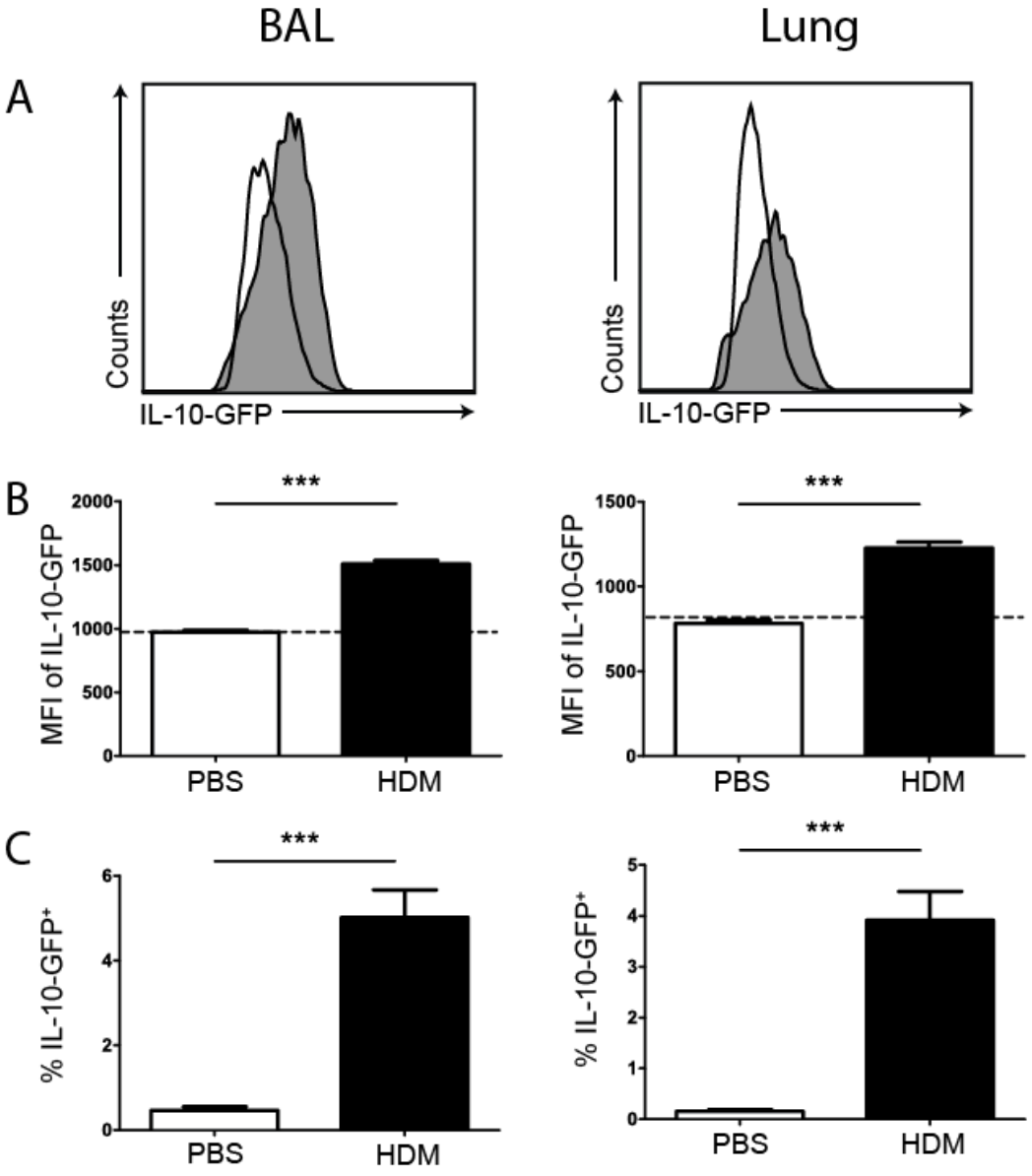

Figure 22. AMs assume an IL-10^{+} phenotype in the BAL and lung tissue following intermediate-term HDM exposure. Male and female Vert-x mice were subjected to intermediate-term (5 wks) PBS or HDM exposure as shown in Chapter 2, *Fig 2*. **(A)** Representative histogram plot of IL-10-GFP expression in CD11c^{+} AMs from PBS control (unshaded) or HDM (shaded) mice. **(B)** MFI of IL-10-GFP expression in CD11c^{+} AMs from PBS control or HDM mice. Dotted line represents negative control (GFP^{-} AMs from a WT mouse). **(C)** Frequency of IL-10-GFP^{+} macrophages expressed as % total AMs from PBS control and HDM mice. Data represent mean ± SEM values (combined for male and females); n = 5-7 per group. ***$p < 0.001$

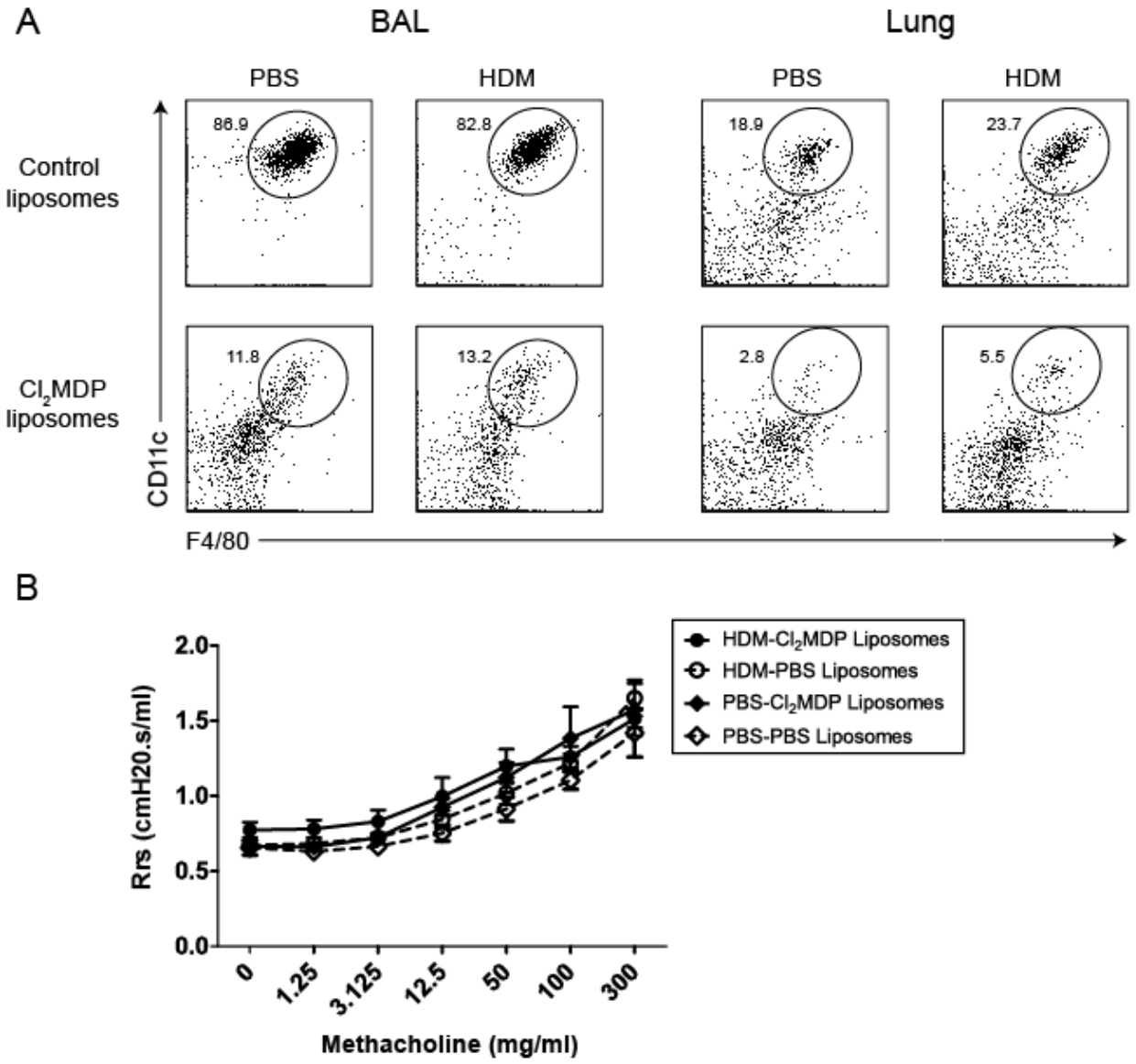

Figure 23. AM depletion does not deter tolerance formation against HDM with long-term exposure. Mice receiving either long-term i.n. PBS or HDM were administered control or clodronate liposomes to deplete AMs as shown in Chapter 2, *Fig 4*. **(A)** Representative plots of AM depletion efficacy in live, $CD45^+$ cell populations in BAL and lungs of PBS- and HDM-instilled mice. **(B)** Mice were tracheostomized, mechanically ventilated, and subjected to methacholine challenge. Changes in Rrs responses to increasing doses of aerosolized methacholine were determined using the flexiVent system (SCIREQ). Data represent mean ± SEM values; n =5 per group. For statistical purposes, group comparisons were based on area under the curve (AUC) measurements. No statistical differences were observed.

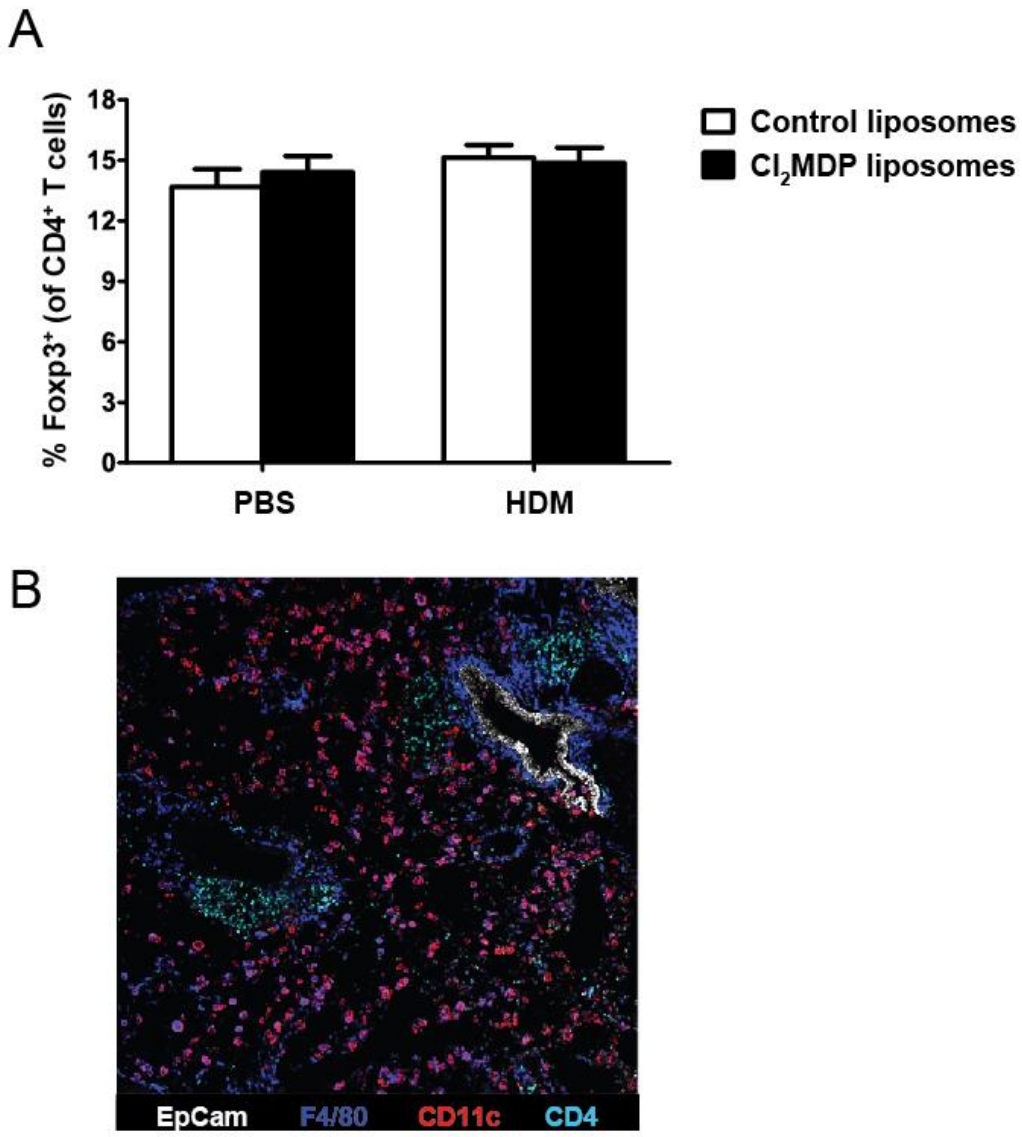

Figure 24. Long-term HDM-exposed AMs do not influence Foxp3^{+} Treg generation in the HLN or lung tissue. **(A)** Long-term HDM or PBS mice were subjected to Cl_2MDP or control liposome treatment as described in Chapter 2, *Fig 4*. Total percentages of HLN Foxp3^{+} Tregs of all CD4+ T cells were determined. Data represent mean ± SEM values; n = 5 per group. No statistical differences were found between groups. **(B)** Lung tissue sections (n=2) from long-term HDM mice stained with fluorescently-conjugated antibodies were examined via confocal microscopy. A representative image is shown above. 10X magnification.

CHAPTER 5

DEVELOPING A MURINE MODEL OF ALLERGEN-SPECIFIC IMMUNOTHERAPY FOR THE TREATMENT OF HOUSE DUST MITE-INDUCED ASTHMA

Introduction

Allergic asthma is a chronic lung disorder that affects 300 million people worldwide. Annual healthcare expenditures for asthma exceed $50 billion, yet current pharmacologic agents for asthma are primarily directed at symptom relief and have limited curative potential and poor prospects for reducing disease prevalence. Allergen-specific immunotherapy (ASIT) is currently the only etiologic therapy for treatment of atopic disorders. By altering the immune response to the triggering allergen, ASIT has been shown to provide long-lasting relief of disease symptoms and improve quality of life in asthma patients. Although the detailed mechanisms by which ASIT exerts its effects are actively being investigated, most studies have been conducted in humans and thus have been unable to evaluate the specific immunologic and physiologic consequences of ASIT within the lungs. Moreover, the few murine models of ASIT for treatment of allergic asthma have been conducted using human-irrelevant allergens such as OVA. Our limited understanding of the mechanisms underlying the effects of ASIT has been a barrier to the development of evidence-based clinical practice guidelines and universally effective ASIT for asthma.

Evidence supports the role of peripheral $Foxp3^+$ Tregs in the clinical efficacy of ASIT[128,214-218], although the mechanism by which these Tregs are induced in asthmatic patients who have successfully undergone ASIT is not well-characterized. Clinical trials have demonstrated that ASIT increases IL-10

production by peripheral monocytes and local macrophages, as was shown in nasal biopsies of patients with seasonal rhinitis[129,130]. However, the contribution of IL-10$^+$ alveolar macrophages (AMs) in patients with asthma has not previously been examined since the site of inflammation (i.e. lung compartments), as mentioned above, cannot be easily accessed in human subjects. Our laboratory has previously shown that AMs from HDM-tolerant mice that produce high levels of IL-10 are capable of inducing Foxp3$^+$ Tregs *in vitro* (Chapter 4, *Fig 21*). However, the contribution of these cells to ASIT and their role on local Foxp3$^+$ Treg induction in the context of HDM-ASIT are not currently known.

The goal of this study was to develop a clinically-relevant murine model to investigate both the specific effects of ASIT on HDM-induced AAD and the potential role of IL-10$^+$ AMs in mediating the clinical benefits of ASIT. Results from this study suggest that biweekly, subcutaneous ASIT with a standardized HDM extract following development of AAD attenuates severity of AAD, with specific reductions in airway eosinophilia, perivascular/peribronchiolar lung inflammation, mucus production, and Th2 cells. However, these clinical benefits of ASIT appeared to occur independently of specific increases in Foxp3$^+$ Tregs or IL-10$^+$ AMs at the site of inflammation.

Results

Short-term ASIT was unable to reproducibly decrease eosinophil frequency in HDM-exposed mice

In order to determine whether ASIT attenuates severity of HDM-induced AAD, short-term HDM mice were administered subcutaneous ASIT with a standardized HDM extract biweekly for 3 weeks at either a low dose or high dose while continuing to receive i.n. HDM (Chapter 2, *Fig 5A*). No adverse reactions in response to ASIT were noted in any mice for the duration of the study.

Although ASIT did not reduce total number of leukocytes in the BAL relative to mice receiving control animals, BAL eosinophil frequency was attenuated in a dose-dependent manner (*Fig 25A*) with compensatory increases in macrophage frequency (*Fig 25B*). However, when these protocols were tried three additional times, no effect of low dose or high dose ASIT on BAL eosinophils was observed, suggesting that short-term ASIT does not reliably attenuate AAD.

Long-term ASIT reduced BAL eosinophilia in HDM-exposed animals

A possible reason why short-term ASIT was not able to repeatedly attenuate AAD is because mice may not adequately develop tolerance to HDM with only six ASIT injections; longer courses of ASIT are likely required for this effect to occur. In order to afford more ASIT injections without risking spontaneous development of tolerance with continuous, intranasal HDM exposure, a $DiscR^{+}$ HDM model was utilized (Chapter 2, *Fig 3*) in which mice are rested and rechallenged to prevent full resolution of BAL eosinophilia (Chapter 3, *Fig 11A*). This long-term model of ASIT is shown in Chapter 2, *Figure 5B*.

Both low and high-dose ASIT significantly reduced total leukocyte counts (55% reduction; *Fig 26A*) in the BAL relative to HDM-Control animals. Non-significant reductions in total leukocyte counts were also observed in the lung with low-dose ASIT (21% reduction) and high-dose ASIT (49% reduction), however, reductions in total HLN leukocytes were only observed with high-dose (41% reduction) and not low-dose ASIT (*Fig 26A*).

In addition to total leukocyte counts, both low- and high-dose ASIT were associated with similar reductions in frequency (~45% reduction; *Fig 26B*) and number (~75% reduction; *Fig 26C*) of eosinophils in the BAL relative to control animals. Although these reductions were non-significant, it is likely that these findings would be statistically significant if group sizes were increased. Macrophage morphology (e.g. frequency of multinucleated cells) was not noted to be significantly altered with ASIT (*data not shown*) despite the fact that compensatory increases in macrophages were also noted with ASIT (*Fig 26B*).

ASIT attenuated inflammation and mucus production in the lungs of HDM-exposed animals

In HDM-Control mice, large perivascular and peribronchiolar inflammatory clusters were noted diffusely throughout the lungs and averaged from 3-5 in number per 10X field (*Fig 27A; top left*). ASIT reduced both the size and number of these clusters per 10X field, with low-dose ASIT (*Fig 27A; top center*) having a greater effect than high-dose ASIT (*Fig 27A; top right*). Nevertheless, both low-dose

ASIT and high-dose ASIT showed significantly reduced inflammation scores compared to control mice when blindly scored (*Fig 27B*).

A large percentage of airways in HDM-Control mice contained ring-like structures of PAS-positive cells, suggesting exacerbated mucus production (*Fig 27A; bottom left*). Although several of these PAS-positive ring-like structures were also seen in the airways of low dose ASIT mice, their frequency was greatly diminished (*Fig 27A; bottom center*). Intriguingly, high dose ASIT mice showed an even further reduction in frequency of PAS-positive airways, with the majority of these airways consisting of punctate PAS staining rather than full ring-like structures (*Fig 27A; bottom right*). Although blinded scoring results did not support a significant decrease in mucus production in the airways of high dose ASIT mice relative to HDM-Control mice (*Fig 27C*), this trend was certainly noticeable on histology. Together, these findings suggest that subcutaneous ASIT decreases both inflammation and mucus production in the lungs, with low-dose ASIT having a drastic effect on number and size of inflammatory clusters and high-dose ASIT more greatly affecting mucus production.

ASIT attenuated serum IgE levels in HDM-exposed animals

As expected, serum IgE levels were comparably elevated across groups after short-term HDM instillation and before initiation of ASIT (*Fig 28*). In HDM-Control animals, $DiscR^{+}$ administration of i.n. HDM was associated with a mean 10-fold increase in IgE levels over the 11 week time course (pre- vs post- ASIT), although the variation in IgE levels between mice was quite high. Total IgE levels were greatly attenuated with high-dose ASIT, as they were only 2.5-fold higher at the end of the 11 week course of i.n. HDM than in pre-ASIT mice. This effect was even more pronounced with low-dose ASIT; in these mice, IgE levels post ASIT were only 1.1-fold higher than levels pre-ASIT. Although it should be noted that the IgE levels observed in low-dose and high-dose ASIT mice were not significantly different from HDM-Control animals due both to small group sizes and high variability in the control group, these levels were 73% and 35% reduced, respectively, post-ASIT.

ASIT attenuated AAD-associated inflammation but did not affect AHR

In order to determine whether the reduction in inflammation associated with ASIT affected AHR, mice were subjected to methacholine challenge and lung resistance values were determined. No differences were observed between resistance curves of HDM-Control, HDM-Low Dose, and HDM-High Dose mice following long-term ASIT (*Fig 29A*). However, even HDM-Control animals did not demonstrate significant AHR with higher doses of methacholine, suggesting that the reduction in AHR associated with continuous, long-term HDM exposure (Chapter 3, *Fig 10*) begins prior to 11 weeks and can be observed in $DiscR^+$ animals as well. Thus, in order to determine whether AHR is truly affected by ASIT, similar studies were conducted in short-term ASIT mice after 5 weeks of i.n. HDM exposure, when AHR has been shown to peak (*Fig 10*). Although HDM-Control animals did demonstrate AHR as expected, neither low-dose ASIT nor high-dose ASIT was able to attenuate these AHR responses (*Fig 29B*), suggesting that short-term ASIT is not sufficient to improve pulmonary function.

ASIT attenuated Th2-associated inflammation but did not affect regulatory leukocyte populations

Decreased BAL eosinophilia and IgE titers suggested that ASIT may reduce Th2-based inflammatory responses. In order to determine this, frequency of T1-$ST2^+$ (IL-33R; a marker of Th2 cells that is important for their effector function[219]) CD4 T cells in the lung tissue was examined. ASIT was associated with dose-dependent decreases in Th2 frequency and number that was significantly attenuated relative to HDM-Control animals with high-dose ASIT (*Fig 30*). This suggests that a major mechanism by which ASIT may attenuate AAD is via reductions in Th2 effector cells, a major player in HDM-induced asthma.

Another mechanism by which ASIT has been thought to benefit patients with asthma is by skewing the balance between effector and regulatory cells in favor of the latter. Surprisingly, no differences were observed in levels of $Foxp3^+$ Tregs in either the lung, HLN, or blood of low-dose or high-dose ASIT animals relative to HDM-Control animals (*Fig 31A*) despite the fact that these cells have been associated with the development of tolerance to long-term HDM exposure (Chapter 3, *Fig 13*). The

appearance of IL-10^{+} AMs in the BAL and lung have also been associated with development of tolerance to long-term HDM exposure (Chapter 3, *Fig 15*). Although some production of IL-10 in AMs was observed in all three groups following DiscR^{+} exposure as was previously observed (Chapter 3, *Fig 16*), no increases in IL-10 production by AMs was noted with either low-dose or high-dose ASIT (*Fig 31B*), suggesting that these cells do not play a significant role in the attenuation of allergic asthma following ASIT.

Discussion

Despite the fact that ASIT has been utilized for over a century, the mechanisms by which subcutaneous therapy benefits patients with asthma remain incompletely understood. This lack of knowledge is reflected by the large variety of ASIT protocols, significant variability in patient responses to ASIT, and the overall hesitance of many clinicians to even recommend ASIT to their asthmatic patients. To better understand the mechanisms of ASIT, we developed and characterized a clinically-relevant murine model of both short-term and long-term ASIT for treatment of HDM-induced allergic airway disease. For these studies, we utilized a standardized HDM extract that is currently administered to asthma patients in the University of Connecticut Health Center ENT clinic.

Previous studies in murine models of subcutaneous ASIT have demonstrated that ASIT suppresses cardinal features of chronic AAD, including airway eosinophilia, Th2 cytokine production, IgE titers, lung inflammation and airway mucus production[122]. Although we observed mild attenuation in BAL eosinophil frequency with short-term ASIT, this finding was not repeatable during future studies and encouraged us to switch to a long-term ASIT model since the clinical efficacy of ASIT has been shown to correlate with duration of therapy[117,220]. Mice subjected to our long-term ASIT protocol experienced many of the aforementioned benefits, although the magnitude of individual effects was often dependent on the dosage of extract administered. A finding of particular interest was the fact that low dose ASIT seemed to suppress perivascular/peribronchiolar lung inflammation on H&E when compared to high dose ASIT, although high dose ASIT reduced total cell counts in all three lung compartments to a

greater extent than low dose ASIT. High dose ASIT was also associated with greater effects on mucus production than low dose ASIT, although the latter showed greater suppressive effects on serum IgE levels. The reasons for these particular dose effects are not well understood, as few studies have delved into the effects of ASIT dose on clinical responses (typically the maximum tolerated dose is administered in humans). However, it is surely appreciated that ASIT outcomes are time- and dose-dependent[221,221], and it has certainly been shown that low-dose ASIT has a more beneficial effect on IgE levels during long-term protocols than high-dose ASIT[222]. In the clinic, ASIT dosing is decided on the basis of wheal responses following skin prick tests, which are difficult to accurately perform in mice. The high dose ASIT protocol utilized in this study (200 AU/injection; 1600 AU/month) was representative of the highest dose administered to patients in the University of Connecticut Health Center ENT clinic, while the low dose ASIT protocol (10 AU/injection; 80 AU/month) was more typical of doses administered to the average patient. However, safety and tolerability studies with high doses of HDM ASIT have shown that 85% of HDM-allergic patients can tolerate maintenance dosages of up to 40,000 AU/month with only grade 1 safety issues[223]. Thus, in the future, it would be interesting to determine whether higher doses of HDM ASIT using our long-term protocol would be associated with more significant or more repeatable outcomes.

Although long-term ASIT was associated with reductions in many immunological parameters, including airway mucus and Th2 cell numbers, we were surprised to find that ASIT did not have an effect on AHR, as has previously been observed in other murine studies using OVA-ASIT[122]. This was, in part, due to the fact that HDM-Control mice in the long-term ASIT studies did not have significant AHR responses despite high levels of lung inflammation, and so it would have been difficult to further reduce lung resistance in experimental mice. Thus, follow-up AHR studies were done in mice receiving short-term ASIT so that AHR could be examined at the peak of response in HDM-Control mice. Although HDM-Control mice did show evidence of AHR at this time point, neither low nor high dose ASIT was able to reduce this response. Although this may have been attributed to the poor repeatability of beneficial immunological outcomes in our short-term ASIT protocol, it is more likely that the duration of ASIT was

not long enough to observe an effect on AHR using the short-term protocol, as a minimum of 8 injections appears to be necessary for a reduction in AHR in mice challenged with human-relevant (i.e. adjuvant-containing) allergens[222]. Murine studies that provide ASIT with extremely large (>1 mg) doses of OVA without the addition of exogenous adjuvants[122] are more likely to tolerize subjects more effectively and in a shorter time frame than ASIT studies in which HDM extract is used. Moreover, this is one of the major reasons why ASIT requires many months to years to show clinical benefits in human subjects. Thus, future studies should be focused on either increasing the number of ASIT injections during the short-term protocol or challenging mice with fewer i.n. HDM injections during the long-term protocol to avoid downregulation of AHR responses in HDM-Control mice.

One of the major mechanisms by which ASIT is thought to benefit allergic/asthmatic patients is through induction of regulatory leukocyte populations, particularly Tregs. Interestingly, frequency of $Foxp3^+$ Tregs did not increase in either the lung (e.g. lung tissue, HLN) or systemic (e.g. blood) compartments with ASIT. This was surprising, given that ASIT is frequently associated with elevated Tregs patients who have successfully undergone this therapy[124,125] and in most mouse models of ASIT that have observed similar effects on inflammatory hallmarks of asthma[122,123,224]. Moreover, we have shown that tolerance to HDM is associated with an increase in HLN $Foxp3^+$ Tregs (Chapter 3), and studies in mouse models of OVA-ASIT have also shown this increase to occur specifically in the local lymph node[122]. However, murine studies have shown that depletion of Tregs at the time of ASIT treatment reverses the beneficial effects on AHR but not on BAL eosinophilia or serum IgE levels, suggesting that ASIT-mediated tolerance induction requires $Foxp3^+$ Tregs for suppression of AHR but not suppression of airway inflammation. Since ASIT did not significantly suppress AHR responses in our model, this may explain why $Foxp3^+$ Tregs were not elevated in mice that received either low- or high-dose ASIT relative to control animals. Nevertheless, ASIT was still able to mediate many of its benefits without specific increases in $Foxp3^+$ Tregs in this model. Other studies have shown $IL\text{-}10^+$ regulatory T cells to be more important for mediating the benefits of ASIT[122,127] While we have not currently examined

the involvement of Tr1 cells within the context of our model, future directions will include studying this Foxp3$^-$ regulatory subset.

In addition to an increase in IL-10 production by T cells, it has been shown that ASIT also increases IL-10 production by peripheral monocytes and local (nasal) macrophages in patients with seasonal rhinitis[129,130]. In the present study, subcutaneous ASIT did not promote increases in IL-10 production by AMs, suggesting that these cells are not directly involved in ASIT-mediated peripheral tolerance or in the *in vivo* conversion of T cells to regulatory subsets. This correlates with our previous data, which failed to show a critical role for these cells in the induction of peripheral tolerance against HDM despite their presence in the BAL and lungs of HDM-tolerant mice.

Herein, we describe the first clinically-relevant murine model of subcutaneous ASIT for treatment of HDM-induced asthma. Results from this study demonstrate that subcutaneous ASIT attenuates severity of AAD by affecting multiple parameters of allergic inflammation, including total WBC counts in the lung compartments, BAL eosinophilia, lung inflammation, mucus production, IgE responses, and Th2 cell frequency. Further evaluation of this model may yield valuable insight into the clinical mechanisms of ASIT, which may in turn be applied to develop safer, more effective ASIT protocols.

Figures

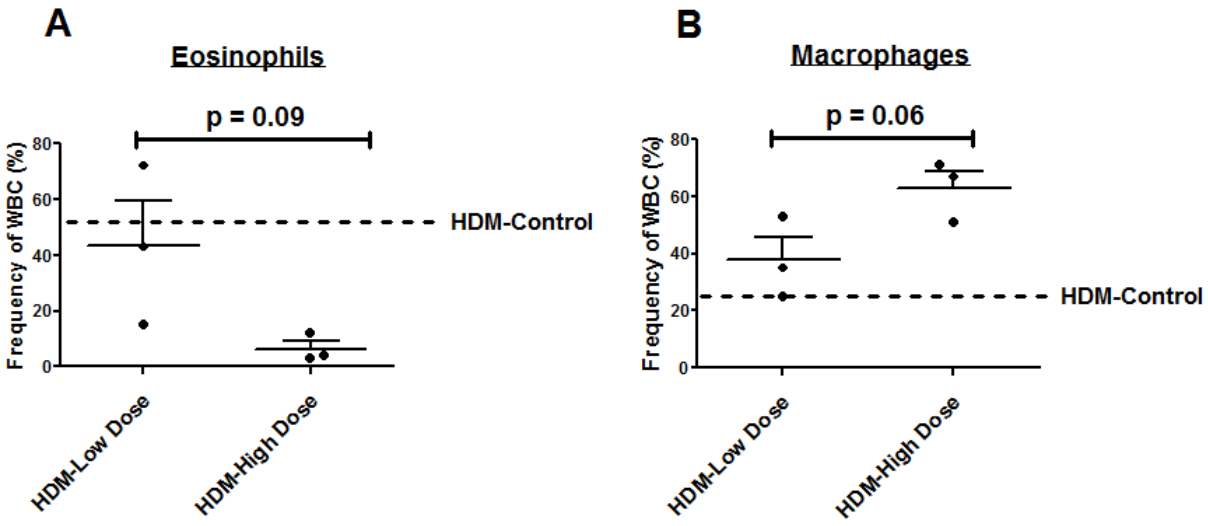

Figure 25. BAL eosinophilia associated with i.n. HDM exposure is reduced by short-term ASIT in a dose-dependent manner. Female C57BL/6 mice were administered i.n. HDM and subjected to short-term ASIT as described in Chapter 2, *Fig 5A*. Cytocentrifuged preparations of BAL cells were stained with May-Grünwald Giemsa and differential analysis was manually performed for frequency of **(A)** eosinophils and **(B)** macrophages. Dotted line represents mean values from HDM-Control mice that received 5 weeks of i.n. HDM and 6 ASIT injections in a time-matched manner. Data represent mean ± SEM values; n = 3 per group.

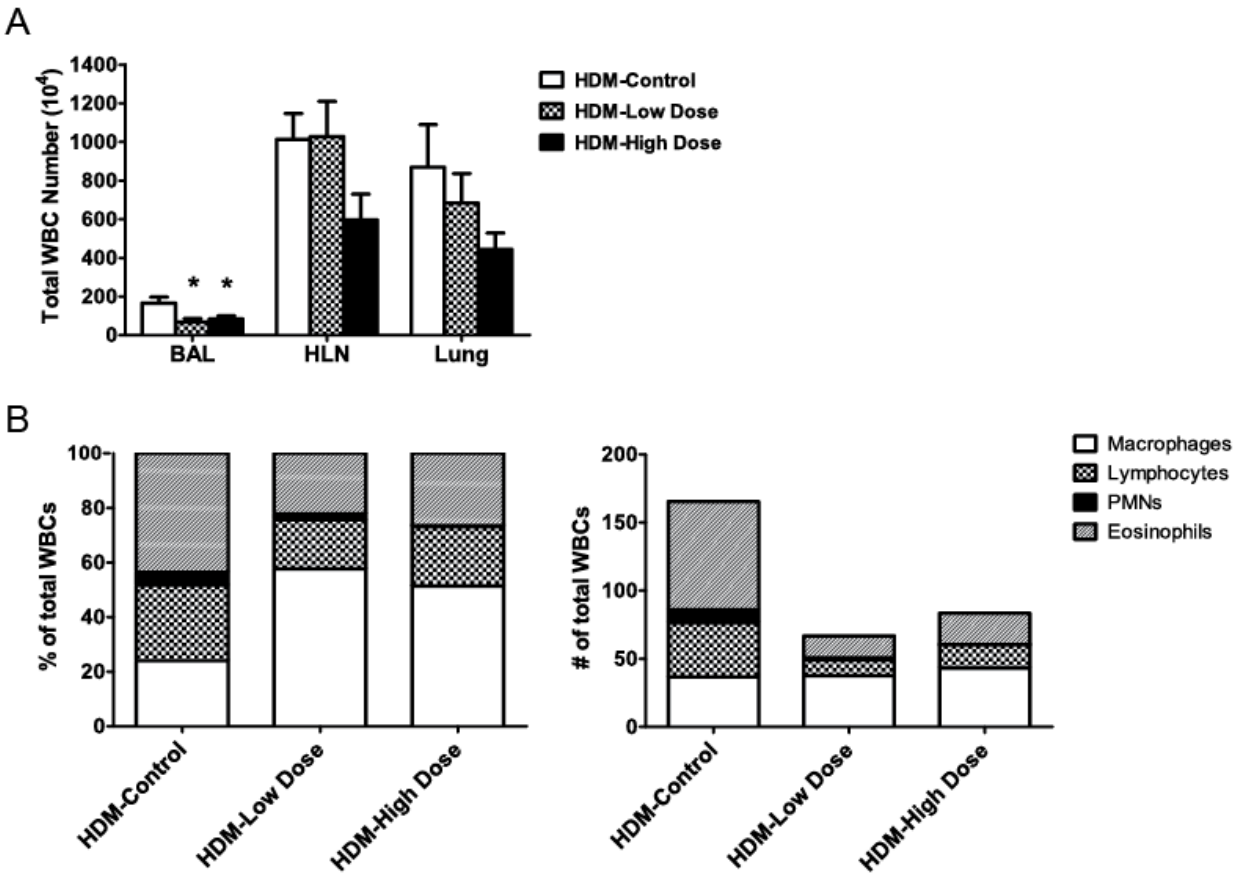

Figure 26. BAL eosinophilia associated with i.n. HDM exposure is attenuated by long-term ASIT. Female Vert-x mice were administered i.n. HDM and subjected to long-term ASIT as described in Chapter 2, *Fig 5B*. **(A)** At sacrifice, total BAL leukocytes were harvested and manually counted on a hemocytometer. **(B-C)** Cytocentrifuged preparations were stained with May-Grünwald Giemsa and differential analysis was manually performed. Data represent mean ± SEM values; n = 5 per group. *p<0.05 vs. HDM-Control.

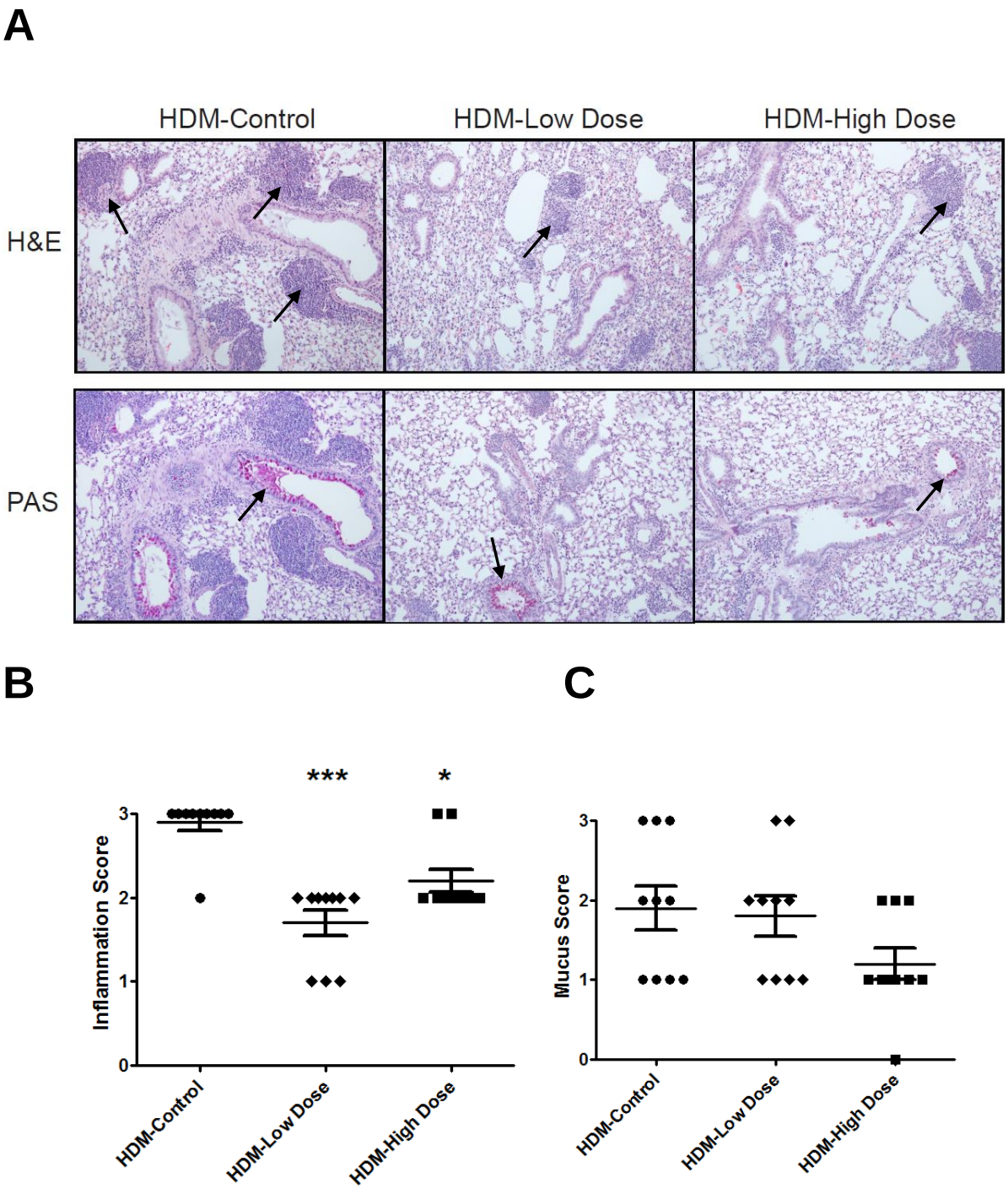

Figure 27. Long-term ASIT attenuates lung inflammation and mucus production associated with i.n. HDM exposure. Formalin-fixed lungs were processed in a standard manner and stained with H&E and PAS. (**Top Row; A)** H&E (X10). Arrows highlight examples of perivascular/peribronchiolar inflammation. (**Bottom Row; A)** PAS (X10). Arrows highlight mucus production by airway goblet cells.

(B) Inflammation scores and **(C)** mucus scores were determined in a blinded fashion on a severity scale from 0-3. Data represent mean ± SEM values; n = 10 per group. *$p<0.05$, ***$p<0.001$ vs. time-matched PBS control.

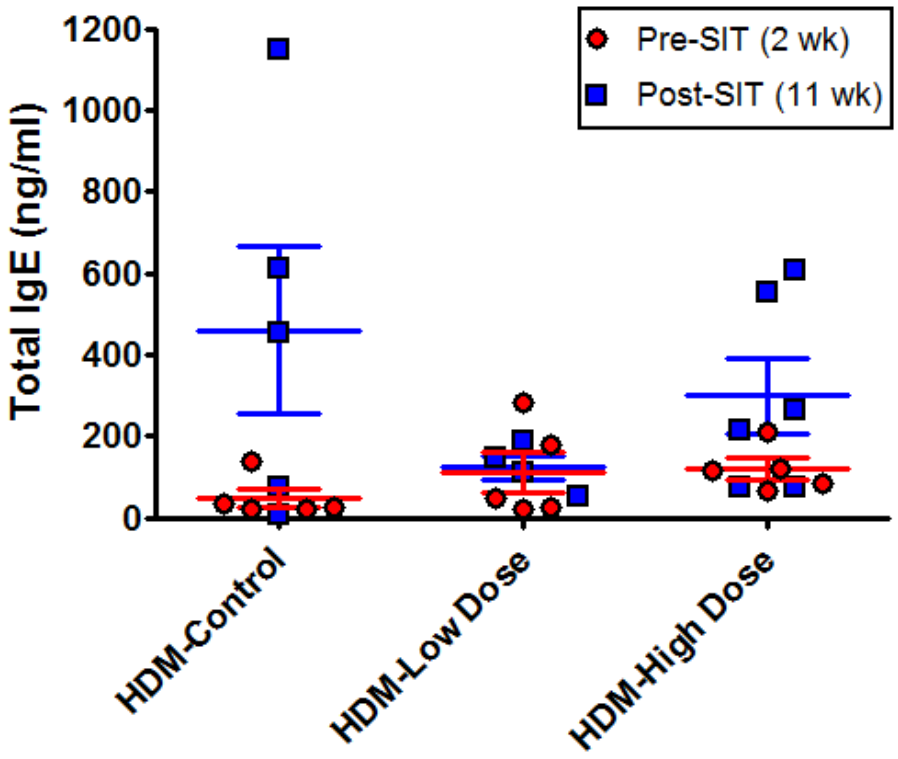

Figure 28. Long-term ASIT following short-term HDM exposure prevents further increases in total serum IgE levels associated with continued i.n. HDM installation. Serum was collected from all animals before the initiation of ASIT (following 2 weeks of HDM exposure; red) and after long-term ASIT (at sacrifice; blue). Total serum IgE levels were determined via ELISA. Data represent mean ± SEM values; n = 5 per group. Statistical comparisons were made between groups pre-ASIT as well as post-ASIT. No statistical differences were observed.

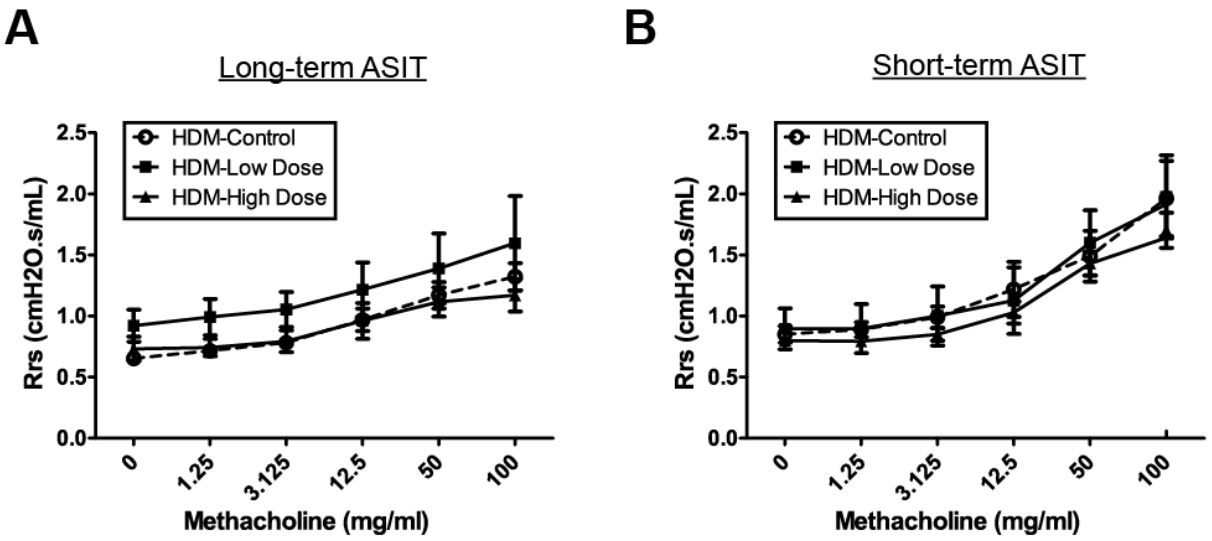

Figure 29. AHR is not suppressed in mice receiving either high-dose or low-dose ASIT. C57BL/6 females who had undergone **(A)** long-term ASIT or **(B)** short-term ASIT were tracheostomized, mechanically ventilated, and subjected to methacholine challenge. Changes in Rrs responses to increasing doses of aerosolized methacholine were determined using the flexiVent system (SCIREQ). Data represent mean ± SEM values; n =5 per group. For statistical purposes, group comparisons were based on area under the curve (AUC) measurements. No statistical differences were observed.

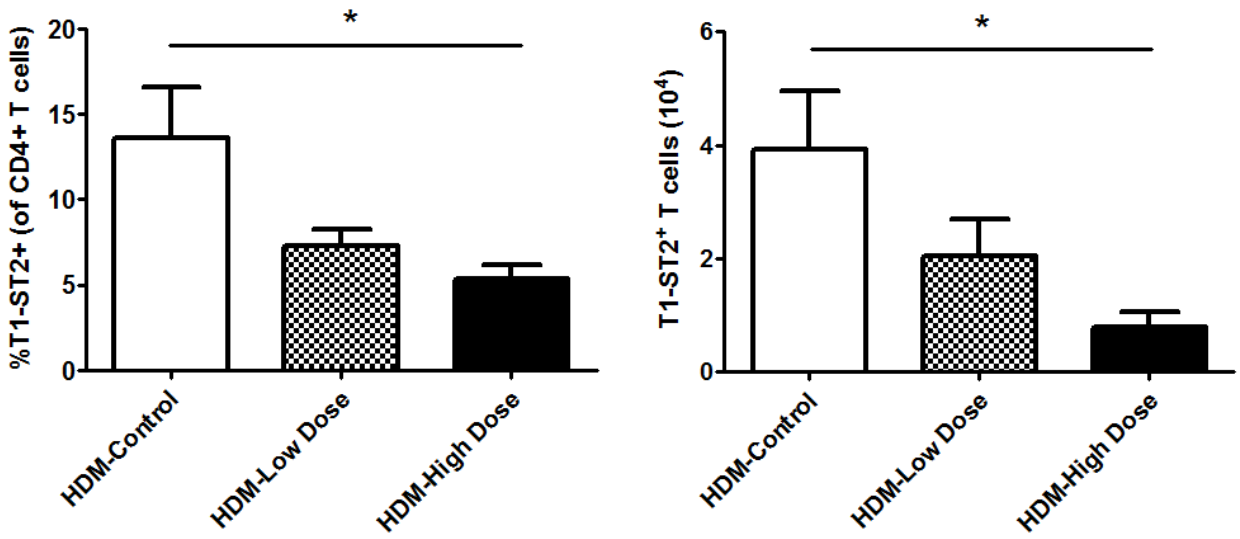

Figure 30. Long-term ASIT reduces lung Th2 cells in a dose-dependent manner. Lung leukocytes were gated on $CD3^{+}CD4^{+}$ T lymphocytes and T1-ST2 expression was examined to determine the frequency and number of Th2 cells. Data represent mean ± SEM values; n = 5 per group. *$p<0.05$ vs. HDM-control.

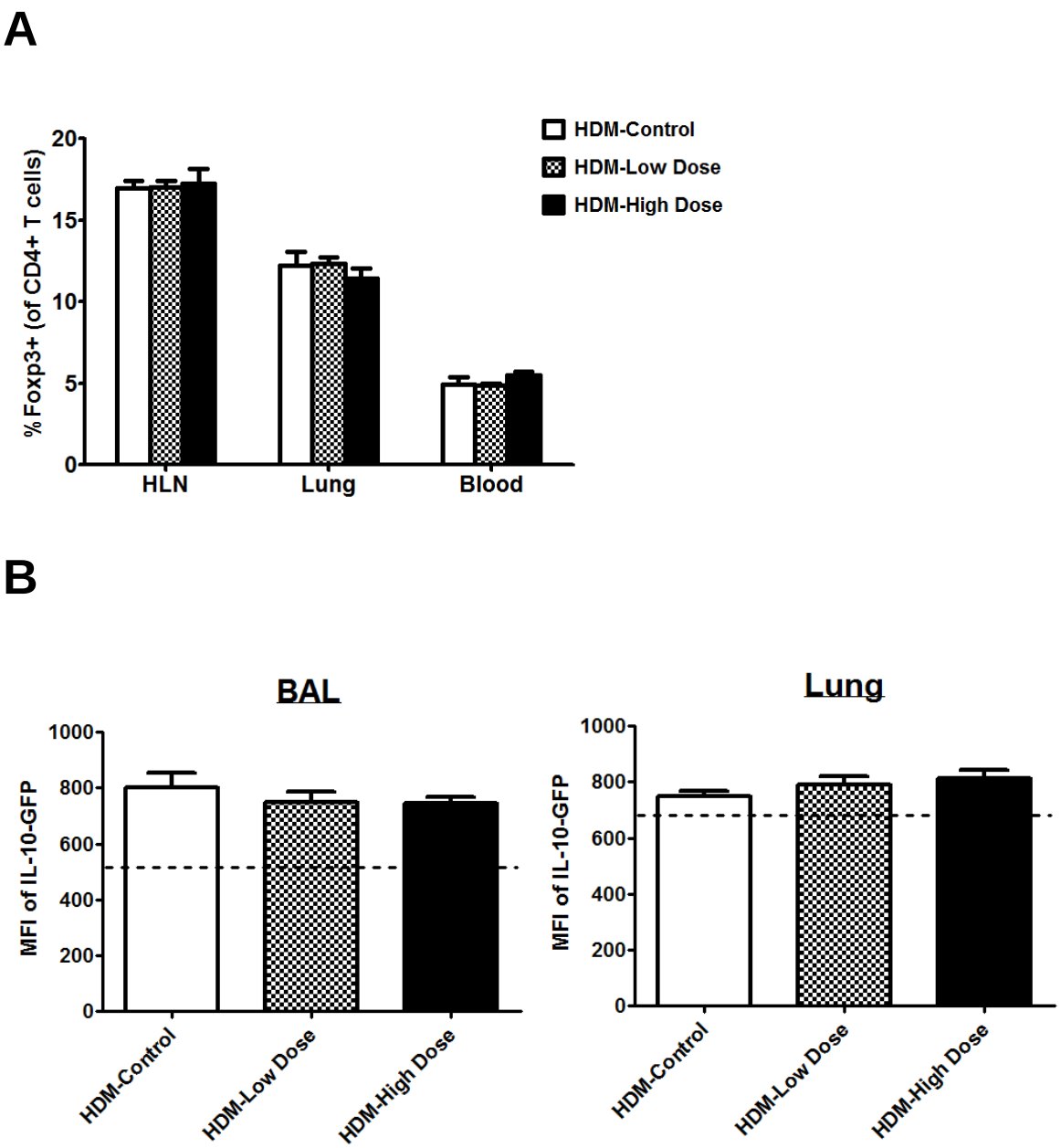

Figure 31. Long-term ASIT is not associated with increases in Foxp3^{+} Tregs or IL-10^{+} AMs. Vert-x mice were subjected to i.n. HDM and long-term ASIT as shown in Chapter 2, *Fig 5B*. **(A)** Cells were gated on CD3^{+}CD4^{+} T lymphocytes and Foxp3 expression was examined. Total percentages of Foxp3^{+} Tregs were determined. **(B)** MFI of IL-10-GFP expression in CD11c^{+} AMs is shown. Dotted line represents negative control (GFP^{-} AMs from a WT mouse). Data represent mean ± SEM values; n = 5 per group. No statistical differences were observed between groups.

CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS

Innovation

Herein, we have provided the first evidence that short- to intermediate- term (2 to 5 wks) intranasal HDM administration promotes development of AAD, while continuous, long-term (11 wks) HDM instillation results in suppression of AAD. These findings were unexpected given that we had previously hypothesized that the immunologic complexity of HDM would inhibit the development of immune tolerance with long-term HDM administration. However, we do believe that this suppressive phase of disease is truly the result of immunologic tolerance due to the presence of immunosuppressive leukocytes (IL-10^+ AMs, $Foxp3^+$ Tregs) at the inflammation site. Moreover, we have shown that IL-10^+ AMs from long-term HDM-exposed animals are capable of inducing $Foxp3^+$ Tregs *in vitro*, a function that has never before been demonstrated with allergen-primed AMs. AMs and Tregs likely cooperate to suppress the features of AAD, although their specific roles in this long-term HDM model have not yet been teased apart (*Fig 32*).

Few studies have examined the effects of long-term HDM exposure on the inflammatory response and even fewer have studied this in the context of tolerance. Thus, this HDM model has tremendous utility for exploring the mechanisms governing immune regulation against complex aeroallergens. Continued investigation into this clinically-relevant, biphasic mouse model of AAD may provide successful approaches for tolerance induction in individuals with asthma.

Future directions and considerations

While these studies have certainly yielded exciting results, there are many more questions that are left to explore:

What does suppression of HDM-induced AAD indicate about the pulmonary state of patients without evidence of AHR?

The HDM-induced AAD model has been extensively utilized among scientists interested in the mechanisms that promote development of asthma; however, studies in long-term HDM models have been few and far between. Our laboratory is the first to demonstrate that long-term HDM exposure can promote a state of immunologic tolerance. However, the suppression of disease that occurs as a consequence of this tolerance is quite unlike the phenomenon of LIT that we have observed with long-term OVA exposure. Despite the fact that both are associated with elevations in Tregs in the HLN (suggesting that some form of active tolerance is certainly responsible for both phenotypes), HDM-associated suppression of AAD is marked by persistent perivascular and peribronchiolar mononuclear lung inflammation, whereas OVA-LIT lungs do not show any evidence of inflammation. Thus, the big question that remains to be answered from these studies is whether the suppression of AAD responses (namely eosinophilia and AHR) suggests absence of disease in the presence of residual inflammation or progression to a different stage or type of lung disease that is no longer signified by a Type I hypersensitivity response. There are several lines of evidence to suggest that the latter is indeed the case, including the fact that IgG1 levels become significantly elevated over the course of HDM exposure. Although not previously discussed, we have also performed histopathologic analysis on lungs from 22 week HDM-exposed mice that demonstrate development of alveolitis in addition to peribronchiolar and perivascular infiltrates despite continued suppression of AHR (*Fig 33*). Could this long-term HDM model signify progression from an asthma-like phenotype to hypersensitivity pneumonitis, two closely related diseases? The latter is marked by a Type IV hypersensivity reaction in which repeated exposure to antigen leads to acute mononuclear alveolitis, intersitital lung infiltration, elevated IgG levels and a

granulomatous-like reaction[225], all of which are present following 11 to 22 weeks of continuous, HDM exposure. The evidence against this theory is the fact that hypersensitivity pneumonitis is associated with pulmonary fibrosis, which surprisingly is not evident at any stage within this 2-22 week HDM model. However, fibrosis can be a slow and insidious process and thus may not be evident until HDM exposure is prolonged for an extreme length of time.

Regardless of whether AAD progresses to hypersensitivity pneumonitis within the context of this model or not, *what is the significance of persistent inflammation within the lungs despite reductions in Th2 inflammatory patterns*? We have speculated that persistent mononuclear inflammation in spite of attenuated mucus production and AHR may be attributed to the tremendous antigenic complexity of HDM[141,177]. It would be valuable to perform additional studies with heat-inactivated HDM to specifically reduce the activities of LPS and various proteolytic enzymes. If this prevented the persistence of lung inflammation with tolerance, one may simply credit the discrepancies between our OVA and HDM models to the fact that these allergens differ so much in their antigenic compositions. However, the possibility exists that this inflammation is truly representative of the lung status in asthmatic patients who have regained tolerance through ASIT or other forms of therapy. In this regard, it is intriguing to speculate that potential subclinical lung inflammation (in the absence of airway eosinophilia, methacholine AHR, or asthma symptoms) could play a role in the relapse of asthma in 40-50% of remitted individuals in later adulthood[183-185]. Yet a second possibility exists that the inflammatory cells observed in the lungs of tolerant mice are primarily regulatory in nature, despite their concerning appearance on histopathology. We have supported this theory by demonstrating a regulatory role for AMs in the lungs of tolerant mice. Follow-up experiments should focus on the functional attributes of other major cell types (e.g. Teff) in the lungs of tolerant mice to determine whether these cells have the same potency (e.g. proliferative ability, Th2 cytokine secretion) as those from diseased mice.

Do AMs truly have a role in tolerance against inhaled allergens?

Although the data presented within this dissertation suggest that AMs do not play an essential role in tolerance induction, additional studies must be done to conclusively rule this possibility out based on the fact that our macrophage depletion techniques had some associated limitations (see *Chapter 4*). The issues involved with using clodronate liposomes to deplete alveolar macrophages could potentially be avoided by using CD11c-DTR mice, although this is also not an ideal option because of the added possibility of simultaneous DC depletion (a fine example of the fact that innate immune technology is not nearly as developed as that for adaptive immune cells). However, the bigger limitation in our studies was the fact that IL-10^+ AMs were not able to be specifically depleted, thus preventing us from segregating their potential anti-inflammatory effects among the total AM population. This question may be better addressed in the future by sorting IL-10^+ AMs from tolerant mice and adoptively transferring them intratracheally into naïve or diseased mice to determine their effects on the kinetics of AAD.

If AMs do play a role in tolerance induction, where does this occur? Previous literature suggests that the induction of Tregs by AMs is not likely to occur in lymph nodes, as AMs do not migrate to the HLN in large numbers[103]. This finding was supported by our *in vivo* AM depletion studies. However, we have shown a steady increase in HLN Treg numbers over the course of the model, begging the question of where these Tregs come from. Based on results from prior studies, Treg induction by AMs can occur in the lung tissue[103]. Our data support this finding, as lung AMs were able to induce Tregs *in vitro,* although we have not seen an accumulation of Tregs in the lungs of HDM-tolerant mice relative to mice with signs of AAD. Nevertheless, we have observed that Tregs are certainly being generated in the lungs of HDM-treated mice relative to control animals at all time points. *Are Tregs generated in the lungs of HDM-exposed mice in vivo, from where they can then migrate to the HLN to dampen Th2 responses?* It has indeed been shown that Tregs can migrate sequentially from inflamed tissues to draining lymph nodes during an immune response, a movement essential for optimal Treg suppression[226]. Along these lines, it would be interesting to intratracheally transfer Tregs from HDM-tolerant mice into congenically-mismatched hosts to determine whether they traffic to the HLN upon HDM sensitization/challenge.

By what process(es) do AMs convert T cells into Tregs? Our confocal imaging data did not demonstrate any direct interactions between AMs and $CD4^+$ T cells in the lungs. Moreover, *in vitro* data show that AMs from long-term HDM mice are not a major source of TGF-β despite the fact that they are able to induce more $Foxp3^+$ Tregs than AMs from short-term HDM or time-matched PBS control animals. *Do AMs from HDM-tolerant mice provide a critical soluble mediator that enhances Treg induction without the need for cell-cell interactions?* This possibility should be tested by performing Treg induction assays using a transwell system to prevent direct AM-Teff contact. Furthermore, *might there be other mechanisms in addition to IL-10 by which these AMs mediate their tolerogenic effects?* Retinoic acid has been shown to increase Tregs by enhancing TGF-β signaling via Smad3 phosphorylation[227]. Multiple studies have shown that AMs can induce Tregs in a retinoic acid-dependent manner[98,103], and so additional Treg induction assays should be performed in the presence of retinoic acid inhibitors. However, this targeted approach may not be the most efficient way to identify critical differences between AMs from long-term and short-term HDM mice. Next steps for this project should center on performing RNA-sequencing analysis of IL-10^+ and IL-10^- AMs throughout the various stages of the model in order to cultivate a better understanding of the specific changes occurring within this cell population with tolerance. Not only would this help identify additional mechanistic targets for tolerance, it may yield additional phenotypic markers of these cells that would allow us to track their relevance in genetically modified (i.e. IL-$10^{-/-}$) mice.

Considerations for ASIT: can we improve upon our current clinical protocols?

Herein, we present the first murine model of HDM-ASIT for the treatment of AAD. This model has numerous advantages, including a clinically-relevant study design (i.e. initiation of treatment post development of disease) and utilization of a standardized HDM extract and biweekly dosing schedule comparable to that administered to asthmatic patients. Moreover, studies from this model can be used to more accurately describe the clinical effects of subcutaneous ASIT at the site of inflammation, which cannot easily be accessed in asthmatic subjects.

Despite the tremendous utility of this model, one of the major concerns with its development has been poor repeatability of results. It should be noted that the studies presented in Chapter 5 are representative of the first (and most striking) experiments conducted for both short- and long-term ASIT studies. Failure of short-term ASIT to show effectiveness after three additional repeats was not surprising, since it is well known that the clinical benefits of ASIT correlate with duration of therapy[228]. We thus adopted the long-term ASIT model in order to improve our odds of observing immunological benefits with therapy. Our long-term ASIT model showed striking differences during our pilot experiments, with changes in almost all major components of allergic inflammation including airway leukocyte numbers, eosinophil levels, IgE levels, lung inflammation, mucus production, and lung Th2 cells. However, when we attempted to repeat these results an additional time using the same clinical protocol, we did not observe such strong differences among groups, raising concerns about how robust most ASIT protocols really are. There is a good deal of evidence to suggest that the benefits of ASIT really do vary from patient to patient. A 2010 Cochrane review of 88 trials in asthma patients receiving ASIT demonstrated that although the combined data showed an overall significant improvement in asthma symptom scores, there was a great deal of heterogeneity among trials across a number of comparisons[229]. Moreover, number needed to treat was typically found to be 3-4 depending on the outcome measured, suggesting that the effects of ASIT a) benefit some but not all patients and b) range dramatically in effectiveness depending on the patient. Interestingly, this heterogeneity was also reflected within our genetically similar mice. Thus, while it will certainly be important for us to experiment with different treatment protocols (e.g. dosage, duration, location, etc.) in the future to find one that is both effective and robust, murine studies will be limited across the board to understand why certain subjects respond to ASIT while others do not. Human studies will be of more help in this arena. More effort should be expended to stratify subjects according to their asthma type and immunologic profile so that these parameters can be directly correlated with ASIT outcomes in these patients. In addition, more studies are required in humans to determine the most effective treatment regimen (e.g. dosage, number of injections per week, route of

injection) for each common allergen, as these protocols can vary drastically from clinical site to clinical site.

In addition to experimenting with various ASIT protocols, there are several other key immunologic parameters that have left to be explored in this model. *What is the status of HDM-specific IgE and IgG1 within the serum and within the airways? How does the ratio of Th2 to Th1 cytokines in the BAL and lung tissue change with ASIT? Is basophil activity altered with ASIT?* It is critical that we can answer these questions using clinically-relevant mouse models, as these compartments are inaccessible in human subjects. Moreover, cytokine and immunoglobulin status in the serum versus the lung compartments do not always correlate, and so it is particularly critical that we do not rely solely on our human data for these answers.

How then does subcutaneous ASIT benefit patients with asthma? Clearly, this mechanism must be different from those involved in traditional tolerance with inhaled allergen exposure. Our data suggest that subcutaneous ASIT is able to effectively decrease Th2 cells in the lungs, but do not indicate an essential role for $Foxp3^+$ Tregs or $IL\text{-}10^+$ AMs in its efficacy. Certainly $IL\text{-}10^+Foxp3^-$ Treg status needs to be examined within our model, as these cells have been implicated in the overall efficacy of ASIT throughout a number of studies. However, the theme of our data (and of the majority of asthma literature) suggests multiple cellular mechanisms are likely at play here. Studies have shown that IgG4 blocking antibody production is confined to IL-10-producing regulatory B cells (Bregs) and that these cells increase with ASIT in allergic patients[230]. We have similarly demonstrated in our murine model of OVA-induced AAD that Bregs have a contributory role in tolerance against inhaled allergens[152]. Thus, the role of these cells in the lungs should specifically be examined in this long-term HDM-ASIT model. Other potential cell types to explore within this model include $CD8^+$ T cells and IL-10-producing DCs. We are also currently in the process of examining whether specific changes in the microbial community within the gut may play a role in the efficacy of subcutaneous ASIT.

Concluding remarks

"I am wiser than this man, for neither of us appears to know anything great and good; but he fancies he knows something, although he knows nothing; whereas I, as I do not know anything, so I do not fancy I do. In this trifling particular, then, I appear to be wiser than he, because I do not fancy I know what I do not know."

-Plato

We exist among a community of researchers that has dedicated itself to identifying a seemingly endless list of cells and cytokines that contribute to the pathogenesis and regulation of asthma- the thought being that we can exploit this knowledge to create *novel* and *specific* pharmacologic agents for asthma. Our laboratory has contributed a unique perspective to this issue through the development of two biphasic AAD models (OVA and HDM) that allow us to study disease progression and resolution over time. As a community, we have successfully reduced disease in our mouse models time and again by either blocking pathogenic cells, antibodies, and cytokines or tipping the balance in favor of regulatory cells. Hundreds of papers have been published in the last decade alone that have yielded promising results for the field of asthma, several of which have proceeded to clinical trials. Yet our clinical trials have, for the most part, been largely disappointing. Is this simply a reflection of a failing animal research paradigm for modeling human disease? There is no denying that mice are not a perfect substitute for humans, but often times we have no option but to conduct our studies in mice. And frequently, we have found enticing results that correlate in mice and humans. We know from our studies that IL-10 is secreted in large amounts by AMs from HDM-tolerant mice and is underproduced in AMs found in asthmatic patients when compared to healthy controls[231]. Moreover, randomized, double-blind trials have shown us that pharmacologic agents capable of reducing clinical symptoms in asthmatic subjects are often linked to statistically significant increases in IL-10[232]. This has made IL-10 and IL-10-producing cells extremely promising targets for the treatment of asthma. Yet when we administered HDM to IL-10-null animals, we

observed no effect on tolerance induction despite the overwhelming literature in favor of the anti-inflammatory role of this cytokine in asthma. What then can we take from these results and why should we not abandon IL-10 all together in search of a more overt target?

The pathophysiology of allergic asthma is based upon a simple concept but in reality, this disease is tremendously complex. While the idea of interrogating a single, specific mechanism in order to find the "magic" cure to a disease is desirable, it is also the major reason why most science does not translate well in the clinic. For one, our scientific process, although capable of providing important insights about the basic mechanisms that govern the human body, fails to take into account the fact that no physiologic or pathologic process exists in isolation: context is important. It is easy to be discouraged by the fact that knocking out IL-10 has "no" effect in our asthma model despite the overwhelming clinical evidence in its favor. However, it is often unclear from such focused studies how additional pathways in the broader system may change in response to alterations in the pathway of interest. In other words, we have no idea how the body will compensate for the absence of IL-10 when, by design, we only ever isolate a single variable at a time. This does not mean that IL-10 is unimportant in the context of asthma, but suggests that IL-10 is only one of several mechanisms aimed at promoting development of immunologic tolerance. I believe that this is why inhaled corticosteroids have remained the current gold-standard treatment for long-term asthma management despite their low specificity. Inhaled corticosteroids are broad-spectrum drugs, and broadly dampening inflammation is the only way to bypass redundancies in the system. Finding specific molecular targets (cytokines, genes, antibodies, etc.) capable of attenuating disease in any significant way is tempting but often difficult to execute. It is for this reason that I advocate that more research be directed towards the field of ASIT, for this etiologic therapy can target multiple parameters of allergic asthma simultaneously without the side effect profile of corticosteroids. Why else has ASIT been our only hope for curing asthma over the last 100 years?

As an aspiring physician-scientist, the process of conducting this research on the involvement of AMs in tolerance against inhaled HDM has provided me with a newfound appreciation for why we have struggled to create targeted therapeutic agents for asthma. Deciphering the mechanisms behind disease is

extremely difficult because one must expect many pathways to be involved, several of which may not be consistent with our neat and tidy paradigms. Thus, I am left to conclude that there are no easy answers to these issues we strive to understand. However, I will end by stating my belief there is benefit in the struggle, regardless of whether or not we generate results that are tantalizing, exciting, or even "publishable". The hole-in-one is rare and many will never achieve it. One has to hope for this most desirable of accomplishments but should not give up learning from every missed swing and every less-than-ideal shot. From that perspective, there is no such thing as a failed experiment. Every bit of data advances our understanding of disease regardless of whether it provides the solution to the problem. Nevertheless, we must also accept the limitations of our deeply focused studies because the full picture is always so much greater than the sum of its parts. I have been humbled by the clinical hurdles that we will face in curing asthma and many other diseases, as my doctoral experience has opened my eyes to how very far we have left to go.

Figures

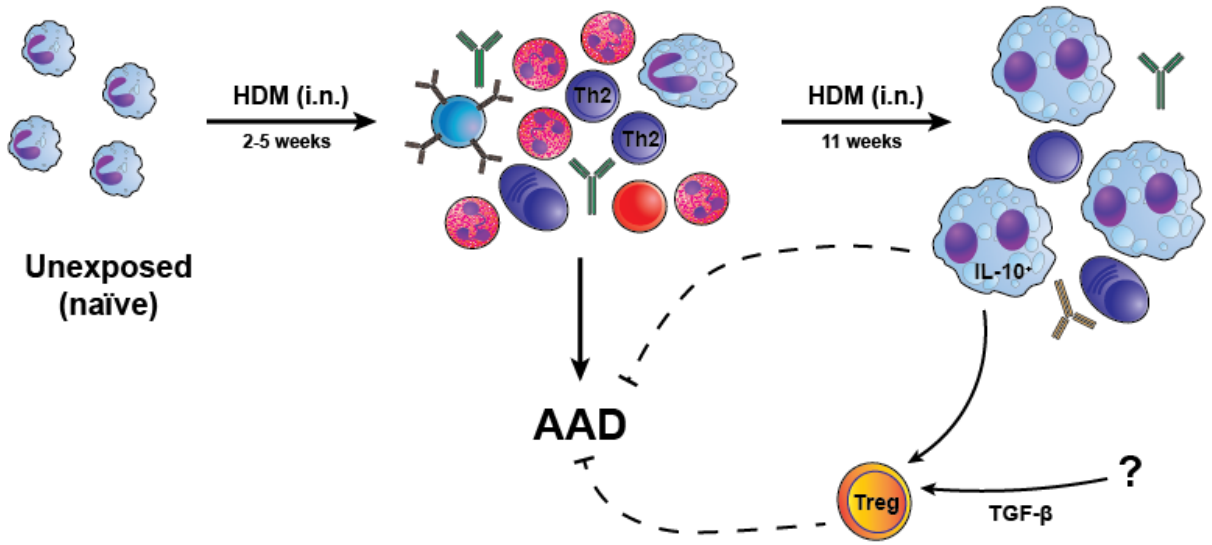

Figure 32. A synthesis of the present findings. 2-5 weeks of continuous, intranasal (i.n.) HDM exposure in a previously naïve mouse promotes Th2 and eosinophilic inflammation, resulting in hallmark signs of AAD. 11 weeks of continuous i.n. HDM administration induces tolerance via development of IL-10^{+} alveolar macrophages, which can, in turn, augment the generation of Foxp3^{+} Tregs in the presence of TGF-β from an unknown source. Tregs and AMs from tolerant mice likely cooperate to suppress features of AAD.

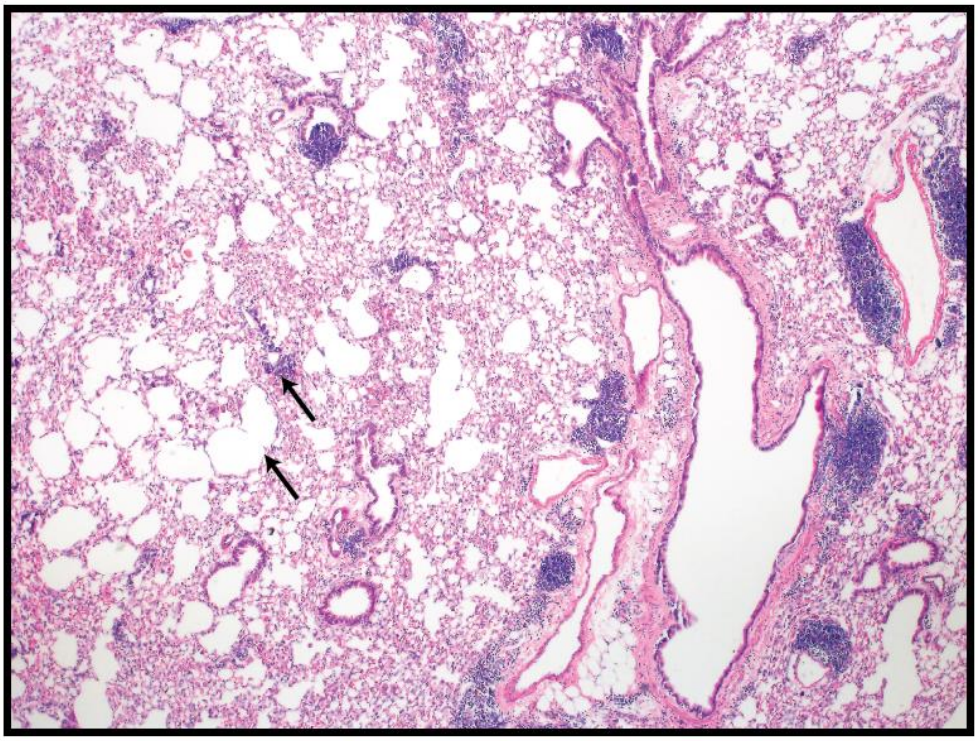

Figure 33. 22 weeks of continuous, intranasal HDM exposure is associated with persistent perivascular, peribronchiolar lung inflammation and development of mild alveolitis. Formalin-fixed lungs were processed in a standard manner and stained with H&E (X10). Arrows highlight examples of distended alveoli that are surrounded by invading leukocytes.

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