Published in final edited form as:

Int Arch Allergy Immunol. 2015 ; 166(4): 243–258. doi:10.1159/000381058.

Long-term exposure to house dust mite leads to suppression of allergic airway disease despite persistent lung inflammation

Sonali J. Bracken^a, Alexander J. Adami^a, Steven M. Szczepanek^b, Mohsin Ehsan^c, Prabitha Natarajan^a, Linda A. Guernsey^a, Neda Shahriari^a, Ektor Rafti^d, Adam P. Matson^d, Craig M. Schramm^d, and Roger S. Thrall^{a,c}

^aDepartment of Immunology, University of Connecticut Health Center, Farmington, CT 06030

^bNeag Comprehensive Cancer Center, University of Connecticut Health Center, Farmington, CT 06030

^cDepartment of Medicine, University of Connecticut Health Center, Farmington, CT 06030

^dDepartment of Pediatrics, Connecticut Children's Medical Center, Hartford, CT 06106

Abstract

Background—Allergic asthma is a major cause of worldwide morbidity and results from inadequate immune regulation in response to innocuous, environmental antigens. The need exists to understand the mechanisms that promote non-reactivity to human-relevant allergens such as house dust mite (HDM) in order to develop curative therapies for asthma. The aim of our study was to compare the effects of short-, intermediate- and long-term HDM administration in a murine asthma model and determine the ability of long-term HDM exposure to suppress allergic inflammation.

Methods—C57BL/6 mice were intranasally instilled with HDM for short-term (2 weeks), intermediate-term (5 weeks) and long-term (11 weeks) periods to induce allergic airway disease (AAD). Severity of AAD was compared across all stages of the model via both immunologic and pulmonary parameters.

Results—Short- and intermediate-term HDM exposure stimulated development of AAD that included eosinophilia in the bronchoalveolar lavage fluid (BAL), pronounced airway hyper-reactivity (AHR), and evidence of lung inflammation. Long-term HDM exposure promoted suppression of AAD, with loss of BAL eosinophilia and AHR despite persistent mononuclear inflammation in the lungs. Suppression of AAD with long-term HDM exposure was associated with an increase in both Foxp3⁺ regulatory T cells and IL-10⁺ alveolar macrophages at the site of inflammation.

Conclusions—This model recapitulates key features of human asthma and may facilitate investigation into the mechanisms that promote immunological tolerance against clinically relevant aeroallergens.

Corresponding author information: Roger S. Thrall, 263 Farmington Avenue, Farmington, CT 06030, (860)679-4118 (phone), (860)679-1047 (fax), thrall@uchc.edu.

The authors declare no conflicts of interest.

Introduction

Allergic asthma is a chronic and debilitating disorder of the airways that impacts nearly 300 million people worldwide [1]. 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 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 immunological tolerance against inhaled, environmental antigens (Ag), resulting in uncontrolled Th2 activation, pulmonary eosinophilia, and airway hyper-reactivity (AHR) [2]. We [3] and others [4–7] 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 ovalbumin (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 [8]. 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.

House dust mite (HDM) is the most causative human allergen worldwide. It is estimated that 50–85% of all asthmatics harbor an allergy to HDM [9, 10], which suggests that the immunomodulatory mechanisms most frequently activated in response to environmental antigens 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 [11, 12]. 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 [13, 14].

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 immunological 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-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.

Materials and Methods

Animals

Female C57BL/6 mice, 6–8 wks of age, were purchased from Jackson Laboratory (Bar Harbor, ME) and conventionally housed in plastic cages with corn cob bedding. In addition, B6(Cg)-*HLI0^{tm1.1Karp}/J* (“Vert-x”) mice (gift of Christopher Karp) 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 [12, 13]. *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 [1]. Total lipopolysaccharide (LPS) content in the HDM extract was 1250 endotoxin units per mg HDM (equal to approximately 0.1 µg LPS/mg HDM [15]; 0.01% contamination).

HDM was administered for five consecutive days, followed by two days of rest, for up to 11 consecutive wks (Fig 1). 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.

BAL 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 as previously described [16]. 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-F4/80 (BM8.1; Tonbo Biosciences, San Diego, CA), anti-CD11b (M1/70; eBioscience, San Diego, CA), anti-CD11c (N418; eBioscience), anti-CD3 (145-2C11; Tonbo Biosciences) and anti-CD4 (RM4-5; Tonbo Biosciences). Samples were stained as previously described [17]. Briefly, cells were washed in PBS containing 0.2% bovine serum albumin and 0.1% NaN₃. Aliquots containing 10⁵–10⁶ 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, Grand Island, NY) 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).

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 [7]. 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 Serum Immunoglobulin Levels

Murine blood was collected via cardiac puncture at the time of sacrifice and used to isolate serum.

For detection of serum HDM-specific IgE, 96-well Nunc MaxiSorp flat bottom plates (Thermo Scientific, Waltham, MA) were coated with 10 µg/ml HDM extract in sodium bicarbonate buffer (pH 9.5) for 16 hours at 4° C. Following washing, plates were blocked for 1 hour at 37° C with BD OptEIA Assay Diluent (BD Biosciences, San Diego, CA). After another washing, serum samples were added as two-fold serial dilutions (range: 1/20 to 1/2560) and allowed to incubate for 1.5 hours at room temperature. Plates were washed

eight times, where after Biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech, Birmingham, AL) was applied, followed by Streptavidin-HRP (BD Biosciences).

Detection of serum HDM-specific IgG₁ was performed as similarly described [12]. Plates were coated with 2 µg/ml HDM extract in sodium bicarbonate buffer for 16 hours at 4° C. Following washing, plates were blocked for 1 hour at room temperature with BD OptEIA Assay Diluent (BD Biosciences). After another washing, serum samples were added as ten-fold serial dilutions (range: 1/20 to 1/200,000,000) and allowed to incubate for 1.5 hours at room temperature. Plates were washed eight times, where after Biotin-SP-conjugated goat anti-mouse IgG Fcγ Subclass 1 specific antibody (Jackson ImmunoResearch, West Grove, PA) was applied, followed by Streptavidin-HRP (BD Biosciences).

For both assays, development was 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.

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. Cells from lung tissue were processed into single cell suspension and lysed via sonication. Cells were spun at 300xg for 5 min at 4° C and supernatant was concentrated ten-fold. 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 nebutol 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). 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.

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.

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 [13], 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 2A). 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 2B). 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 2C), 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 2B). In addition, BAL eosinophil number decreased significantly following long-term HDM exposure when compared to short-term exposure (Fig 2C)). This trend was similar to that observed for lymphocytes, which peaked in frequency (Fig 2B) and number (Fig 2C) 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 2B). Macrophage frequency dropped to 28 ± 4 % in short-term HDM mice as a result of a relative increase in eosinophils and lymphocytes (Fig 2B). 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 2C). Moreover, airway macrophages demonstrated dramatic alterations in their histological appearance over the course of disease (Fig 2D). Whereas macrophages from PBS control mice appeared small, round, and inactive (Fig 2D- upper left) at all time points, those from short-term HDM (Fig 2D- upper right) and intermediate-term (Fig 2D- lower left) HDM-exposed mice were more elongated, vacuolated, and began to show evidence of multinucleation, Fig 2D, E).

Macrophages from long-term HDM mice increased dramatically in size compared to control, short-term HDM, and intermediate-term HDM-exposed macrophages (Fig 2D-lower right). In addition, there was a substantial increase in the frequency (Fig 2E) and number (Fig 2F) 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 3A). 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. Of note, total serum IgE titers in short-term HDM-exposed mice were found to be elevated above levels in time-matched PBS control mice and did not significantly change following intermediate-term or long-term HDM exposure (*data not shown*). HDM-specific IgG₁ levels followed a similar trend as HDM-specific IgE, increasing steadily over the course of HDM exposure and peaking following long-term instillation (Fig 3B).

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 4). 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 INF γ have been shown to inhibit Th2 cytokine functions and allergic eosinophilia when acting on the airway epithelium [18]. Local (BAL and lung tissue) INF γ levels approximated or fell below the limit of detection in HDM-exposed mice at all time points (Fig 4). Intriguingly, INF γ 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 5A, I). Early histological alterations were observed after short-term (2 wks) HDM exposure in the form of small, inflammatory pockets (Fig 5B; short arrow) and increased following intermediate-term (5 wks) HDM exposure in the form of large perivascular and peribronchiolar inflammatory clusters (Fig 5C; 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 5D; long arrow). Although BAL eosinophilia was attenuated in long-term HDM mice (Fig 2C, D), diffuse peribronchiolar and perivascular inflammation in the lung tissue peaked at this time point (Fig 5D; short arrow). Semi-quantitative analysis supported an increase in inflammation above time-matched PBS controls (Fig 5I). 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 5D; 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 5E). 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 5F, G; arrows), a conclusion which was supported by blinded scoring results (Fig 5J). 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 5H; arrows and Fig 5J). 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 6). 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 2B) 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 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 antigens occur at the site of inflammation [17, 19]. 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 7A, 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 2A) but were not increased in long-term HDM mice relative to short-term or intermediate-term HDM mice (Fig 7C).

Foxp3⁺ Treg frequency among total CD4⁺ T cells in the HLN was approximately $14 \pm 2\%$ in PBS control groups and was not significantly elevated with short-term or intermediate-term HDM exposure (Fig 7A, B). However, long-term HDM-exposed mice demonstrated significant increases in HLN Foxp3⁺ Treg frequency ($20 \pm 2\%$) 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 7B). Most intriguingly, these kinetics correlated with resolution of eosinophilia in the airways (Fig 2B, C). Of note, no major differences were observed in levels of CD19⁺ B cells or CD4⁺ and CD8⁺ T cells in any of the lung compartments across the various stages of the model (*data not shown*). These findings suggest that selective expansion of Tregs in the HLN may play a critical role in suppression of AAD following long-term antigen 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 2B, C), we examined changes in M2 alveolar macrophage status (F4/80⁺CD11c⁺ cells; Fig 8A) throughout the course of the model. Production of IL-10 (a hallmark regulatory cytokine produced by M2 macrophages [20]) 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 1. 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 8B). 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 8C). 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 8D). 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⁺CD11c⁺ AMs and not in F4/80⁺CD11c⁻ interstitial lung macrophages (IMs; Fig 8E). 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 2D).

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 ovalbumin (OVA) leads to a biphasic response in which short-term exposure promotes development of AAD that resolves following long-term exposure [3]. 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 [14, 21, 22], 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 TNF α production by dendritic cells and macrophages [23]. Moreover, TNF α has been shown to influence vascular and lymphatic remodeling in mice with sustained airway inflammation [24]. 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 [25], 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 [26]. Although one group has shown that long-term (20 weeks) HDM exposure does not result in resolution of AHR [21], 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 [27]. 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 antigen exposure has been previously demonstrated by our laboratory [28] as well as by others [7]. 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 [29]. 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 IgG₁ responses were also increased over the course of antigen 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 IgG₁ (murine equivalent of IgG4 in humans) in both mice [30] and humans [31]. Murine IgG₁/human IgG4 have been thought to act as blocking antibodies that can compete with IgE for allergen binding. Thus, systemic increases in IgG₁ 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 [32]. 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. INF γ levels remained at or below the limit of detection in local (BAL and lung tissue) compartments. Although INF γ 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 [3]. 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 [3, 33]. 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 [34]. 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 [1, 35].

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 [36]. 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 [37]. 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 [38]. Nevertheless, OVA is a simpler antigen 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 [39] and in airway epithelial cells [40]. 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 [41]. However, paucigranulocytic asthmatics demonstrate hyperresponsiveness to methacholine [41] – 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 [42–44]. 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 [42–44].

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 [45, 46] 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 [45, 47]. 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 [19] and that this induction occurs specifically through interactions with B regulatory cells [17]. Thus, increases in HLN Tregs with long-term HDM exposure may have played an important role in the suppression of AAD.

In addition, 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 [48]. 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 [49], 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 [50, 51]. Additionally, GM-CSF has been shown to promote AM differentiation into multinucleated macrophages [52], and HDM-induced AAD has been shown to be dependent on endogenous GM-CSF production [12]. 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 [53]. 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 [54]. Intriguingly, studies have demonstrated that IL-10⁺ macrophages are critical for the maintenance of Foxp3 expression in mucosal Tregs [55]. 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 [56, 57] and is often decreased in the BAL of asthmatic patients relative to healthy controls [58]. 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 [59]. 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.

In summary, the present study provides the first evidence that 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.

Acknowledgments

This work was supported by NIH grants R01-AI43573 (RST), T32-AI007080 (SJB), and F30-HL122018 (SJB)

Vert-x mice were originally obtained from Christopher Karp at Cincinnati Children's Hospital and generously provided to us by Leo Lefrancois at UConn Health Center. Special thanks are extended to Thiruchandurai Rajan at UConn Health Center for help with analysis of lung pathology.

References

1. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 2011; 32:402–411. [PubMed: 21783420]
2. Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. *Immunol Rev.* 2006; 212:238–255. [PubMed: 16903918]
3. Yiamouyiannis CA, Schramm CM, Puddington L, Stengel P, Baradaran-Hosseini E, Wolyniec WW, Whiteley HE, Thrall RS. Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model. *Am J Pathol.* 1999; 154:1911–1921. [PubMed: 10362818]
4. Faustino L, Fonseca DM, Florsheim EB, Resende RR, Lepique AP, Faquim-Mauro E, Gomes E, Silva JS, Yagita H, Russo M. Tumor necrosis factor-related apoptosis-inducing ligand mediates the resolution of allergic airway inflammation induced by chronic allergen inhalation. *Mucosal Immunol.* 2014; 7:1199–1208. [PubMed: 24569802]
5. Sakai K, Yokoyama A, Kohno N, Hamada H, Hiwada K. Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. *Int Arch Allergy Immunol.* 2001; 126:126–134. [PubMed: 11729350]
6. Swirski FK, Sasic D, Robbins CS, Gajewska BU, Jordana M, Stampfli MR. Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. *J Immunol.* 2002; 169:3499–3506. [PubMed: 12244139]
7. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged inhaled allergen exposure can induce persistent tolerance. *Am J Respir Cell Mol Biol.* 2007; 36:573–584. [PubMed: 17218615]
8. Cates EC, Fattouh R, Johnson JR, Llop-Guevara A, Jordana M. Modeling responses to respiratory house dust mite exposure. *Contrib Microbiol.* 2007; 14:42–67. [PubMed: 17684332]
9. Boulet LP, Turcotte H, Laprise C, Lavertu C, Bedard PM, Lavoie A, Hebert J. Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. *Clin Exp Allergy.* 1997; 27:52–59. [PubMed: 9117881]
10. Nelson RP Jr, DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. *J Allergy Clin Immunol.* 1996; 98:258–263. [PubMed: 8757201]
11. Plantinga M, Guillems M, Vanheerswynghe M, Deswarte K, Branco-Madeira F, Toussaint W, Vanhoutte L, Neyt K, Killeen N, Malissen B, Hammad H, Lambrecht BN. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity.* 2013; 38:322–335. [PubMed: 23352232]
12. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol.* 2004; 173:6384–6392. [PubMed: 15528378]
13. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med.* 2004; 169:378–385. [PubMed: 14597485]

14. Rydell-Tormanen K, Johnson JR, Fattouh R, Jordana M, Erjefalt JS. Induction of vascular remodeling in the lung by chronic house dust mite exposure. *Am J Respir Cell Mol Biol.* 2008; 39:61–67. [PubMed: 18314535]
15. Schaumberger S, Ladinig A, Reisinger N, Ritzmann M, Schatzmayr G. Evaluation of the endotoxin binding efficiency of clay minerals using the *Limulus* Amebocyte lysate test: an in vitro study. *AMB Express.* 2014; 4:1-0855-4-1. [PubMed: 24383578]
16. McNamara JT, Schramm CM, Singh A, Secor ER Jr, Guernsey LA, Lefrancois L, Thrall RS. Phenotypic changes to the endogenous antigen-specific CD8+ T cell response correlates with the development and resolution of allergic airway disease. *Am J Pathol.* 2012; 180:1991–2000. [PubMed: 22452921]
17. Natarajan P, Singh A, McNamara JT, Secor ER Jr, Guernsey LA, Thrall RS, Schramm CM. Regulatory B cells from hilar lymph nodes of tolerant mice in a murine model of allergic airway disease are CD5(+), express TGF-beta, and co-localize with CD4(+)Foxp3(+) T cells. *Mucosal Immunol.* 2012
18. Mitchell C, Provost K, Niu N, Homer R, Cohn L. IFN-gamma acts on the airway epithelium to inhibit local and systemic pathology in allergic airway disease. *J Immunol.* 2011; 187:3815–3820. [PubMed: 21873527]
19. Carson WF 4th, Guernsey LA, Singh A, Vella AT, Schramm CM, Thrall RS. Accumulation of regulatory T cells in local draining lymph nodes of the lung correlates with spontaneous resolution of chronic asthma in a murine model. *Int Arch Allergy Immunol.* 2008; 145:231–243. [PubMed: 17914275]
20. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008; 8:958–969. [PubMed: 19029990]
21. Mushaben EM, Hershey GK, Pauciulo MW, Nichols WC, Le Cras TD. Chronic allergic inflammation causes vascular remodeling and pulmonary hypertension in *BMPR2* hypomorph and wild-type mice. *PLoS One.* 2012; 7:e32468. [PubMed: 22427841]
22. Llop-Guevara A, Colangelo M, Chu DK, Moore CL, Stieber NA, Walker TD, Goncharova S, Coyle AJ, Lundblad LK, O'Byrne PM, Lovric M, Jordana M. In vivo-to-in silico iterations to investigate aeroallergen-host interactions. *PLoS One.* 2008; 3:e2426. [PubMed: 18545674]
23. Fei M, Bhatia S, Oriss TB, Yarlagadda M, Khare A, Akira S, Saijo S, Iwakura Y, Fallert Junecko BA, Reinhart TA, Foreman O, Ray P, Kolls J, Ray A. TNF-alpha from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection. *Proc Natl Acad Sci USA.* 2011; 108:5360–5365. [PubMed: 21402950]
24. Baluk P, Yao LC, Feng J, Romano T, Jung SS, Schreiter JL, Yan L, Shealy DJ, McDonald DM. TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *J Clin Invest.* 2009; 119:2954–2964. [PubMed: 19759514]
25. Sahu N, Morales JL, Fowell D, August A. Modeling susceptibility versus resistance in allergic airway disease reveals regulation by Tec kinase Itk. *PLoS One.* 2010; 5:e11348. [PubMed: 20596543]
26. Tournoy KG, Kips JC, Schou C, Pauwels RA. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy.* 2000; 30:79–85. [PubMed: 10606934]
27. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci USA.* 2001; 98:5175–5180. [PubMed: 11296262]
28. Schramm CM, Puddington L, Wu C, Guernsey L, Gharaee-Kermani M, Phan SH, Thrall RS. Chronic inhaled ovalbumin exposure induces antigen-dependent but not antigen-specific inhalational tolerance in a murine model of allergic airway disease. *Am J Pathol.* 2004; 164:295–304. [PubMed: 14695342]
29. Singh A, Thrall RS, Guernsey LA, Carson WF 4th, Secor ER Jr, Cone RE, Rajan TV, Schramm CM. Subcutaneous late phase responses are augmented during local inhalational tolerance in a murine asthma model. *Immunol Cell Biol.* 2008; 86:535–538. [PubMed: 18458679]

30. Bohm L, Maxeiner J, Meyer-Martin H, Reuter S, Finotto S, Klein M, Schild H, Schmitt E, Bopp T, Taube C. IL-10 and Regulatory T Cells Cooperate in Allergen-Specific Immunotherapy To Ameliorate Allergic Asthma. *J Immunol.* 2015; 194:887–897. [PubMed: 25527785]
31. Lai X, Li J, Xiao X, Liu E, Zhang C, Wang H, Gjesing B, Zhong N, Spangfort MD. Specific IgG4 production during house dust mite immunotherapy among age, gender and allergic disease populations. *Int Arch Allergy Immunol.* 2013; 160:37–46. [PubMed: 22948109]
32. Maggi E. T-cell responses induced by allergen-specific immunotherapy. *Clin Exp Immunol.* 2010; 161:10–18. [PubMed: 20408857]
33. Van Hove CL, Maes T, Cataldo DD, Gueders MM, Palmans E, Joos GF, Tournoy KG. Comparison of acute inflammatory and chronic structural asthma-like responses between C57BL/6 and BALB/c mice. *Int Arch Allergy Immunol.* 2009; 149:195–207. [PubMed: 19218812]
34. Johnson JR, Roos A, Berg T, Nord M, Fuxe J. Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways. *PLoS One.* 2011; 6:e16175. [PubMed: 21283768]
35. Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol.* 2002; 129:1–18. [PubMed: 12372994]
36. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med.* 2009; 15:410–416. [PubMed: 19330007]
37. Taube C, Dakhama A, Rha YH, Takeda K, Joetham A, Park JW, Balhorn A, Takai T, Poch KR, Nick JA, Gelfand EW. Transient neutrophil infiltration after allergen challenge is dependent on specific antibodies and Fc gamma III receptors. *J Immunol.* 2003; 170:4301–4309. [PubMed: 12682266]
38. Schramm CM, Guernsey L, Secor E, Thrall RS. Tolerance induced by chronic inhaled antigen in a murine asthma model is not mediated by endotoxin. *Biochim Biophys Acta.* 2006; 1762:499–501. [PubMed: 16616459]
39. Wang JY. The innate immune response in house dust mite-induced allergic inflammation. *Allergy Asthma Immunol Res.* 2013; 5:68–74. [PubMed: 23450108]
40. Jacquet A. Innate immune responses in house dust mite allergy. *ISRN Allergy.* 2013; 2013:735031. [PubMed: 23724247]
41. Manise M, Holtappels G, Van Crombruggen K, Schleich F, Bachert C, Louis R. Sputum IgE and cytokines in asthma: relationship with sputum cellular profile. *PLoS One.* 2013; 8:e58388. [PubMed: 23555579]
42. De Marco R, Locatelli F, Cerveri I, Bugiani M, Marinoni A, Giammanco G. Italian Study on Asthma in Young Adults study group. Incidence and remission of asthma: a retrospective study on the natural history of asthma in Italy. *J Allergy Clin Immunol.* 2002; 110:228–235. [PubMed: 12170262]
43. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, Cowan JO, Herbison GP, Silva PA, Poulton R. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med.* 2003; 349:1414–1422. [PubMed: 14534334]
44. Komatsu Y, Fujimoto K, Yasuo M, Urushihata K, Hanaoka M, Koizumi T, Kubo K. Airway hyper-responsiveness in young adults with asthma that remitted either during or before adolescence. *Respirology.* 2009; 14:217–223. [PubMed: 19272083]
45. Robinson DS. Regulatory T cells and asthma. *Clin Exp Allergy.* 2009; 39:1314–1323. [PubMed: 19538496]
46. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber CB, Blaser K, Akdis CA. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med.* 2004; 199:1567–1575. [PubMed: 15173208]
47. Taylor A, Verhagen J, Akdis CA, Akdis M. T regulatory cells in allergy and health: a question of allergen specificity and balance. *Int Arch Allergy Immunol.* 2004; 135:73–82. [PubMed: 15340251]

48. Changani K, Pereira C, Young S, Shaw R, Campbell SP, Pindoria K, Jordan S, Wiley K, Bolton S, Nials T, Haase M, Pedrick M, Knowles R. U-BIOPRED Consortium. Longitudinal characterization of a model of chronic allergic lung inflammation in mice using imaging, functional and immunological methods. *Clin Sci (Lond)*. 2013; 125:555–564. [PubMed: 23725401]
49. Anderson S, Shires VL, Wilson RA, Mountford AP. Formation of multinucleated giant cells in the mouse lung is promoted in the absence of interleukin-12. *Am J Respir Cell Mol Biol*. 1999; 20:371–378. [PubMed: 10030834]
50. Helming L, Gordon S. The molecular basis of macrophage fusion. *Immunobiology*. 2007; 212:785–793. [PubMed: 18086379]
51. Helming L, Gordon S. Macrophage fusion induced by IL-4 alternative activation is a multistage process involving multiple target molecules. *Eur J Immunol*. 2007; 37:33–42. [PubMed: 17154265]
52. Lemaire I, Yang H, Lauzon W, Gendron N. M-CSF and GM-CSF promote alveolar macrophage differentiation into multinucleated giant cells with distinct phenotypes. *J Leukoc Biol*. 1996; 60:509–518. [PubMed: 8864136]
53. Iyer SS, Ghaffari AA, Cheng G. Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. *J Immunol*. 2010; 185:6599–6607. [PubMed: 21041726]
54. Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada Calvo F, Henry E, Closset R, Dewals B, Thielen C, Gustin P, de Leval L, Van Rooijen N, Le Moine A, Vanderplasschen A, Cataldo D, Drion PV, Moser M, Lekeux P, Bureau F. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest*. 2009; 119:3723–3738. [PubMed: 19907079]
55. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, Kronenberg M. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol*. 2009; 10:1178–1184. [PubMed: 19783988]
56. Wilson MS, Elnekave E, Mentink-Kane MM, Hodges MG, Pesce JT, Ramalingam TR, Thompson RW, Kamanaka M, Flavell RA, Keane-Myers A, Cheever AW, Wynn TA. IL-13 α 2 and IL-10 coordinately suppress airway inflammation, airway-hyperreactivity, and fibrosis in mice. *J Clin Invest*. 2007; 117:2941–2951. [PubMed: 17885690]
57. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, Berry G, DeKruyff RH, Umetsu DT. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med*. 2002; 8:1024–1032. [PubMed: 12145647]
58. Borish L, Aarons A, Rumblyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol*. 1996; 97:1288–1296. [PubMed: 8648025]
59. John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, Barnes PJ, Chung KF. Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1 α , granulocyte-macrophage colony-stimulating factor, and interferon- γ release from alveolar macrophages in asthma. *Am J Respir Crit Care Med*. 1998; 157:256–262. [PubMed: 9445307]

Figure 1.

Continuous model of HDM-induced AAD. C57BL/6 mice were lightly anesthetized with isoflurane and administered 25 μ g lyophilized HDM extract in 50 μ l PBS via intranasal (*i.n.*) instillation for up to 11 wks as described in *Materials and Methods*. 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 2.

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 Fig 1A. (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 μ m. (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 $\pm$ 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) ^ap<0.05 vs. time-matched PBS control; ^bp<0.05 vs. short-term HDM; ^cp<0.05 vs. intermediate-term HDM.

Figure 3.

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 IgE and IgG1 levels were determined via ELISA. Dual absorbance at 450 nm and 570 nm was calculated over a range of dilutions. Data represent mean $\pm$ 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 4.

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 $\pm$ 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 5.

HDM-exposed mice demonstrated persistent perivascular/peribronchiolar inflammation. Formalin-fixed lungs were processed in a standard manner and stained with H&E and PAS. (**Top Row; AD**) H&E (X4, scale bar = 300 μ m). 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 μ m). 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 $\pm$ SEM values; n = 5 per group.

*p<0.05 vs. time-matched PBS control.

Figure 6.

AHR peaked following intermediate-term HDM exposure but was 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 $\pm$ 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. AUC of All PBS; ^b $p < 0.001$ vs. AUC of long-term HDM; ^c $p < 0.01$ vs. AUC of short-term HDM.

Figure 7.

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 $\pm$ 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 8.

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 Fig 1. **(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 antigen exposure in PBS control (unshaded) or HDM (shaded) mice. Solid, dark grey-lined histograms represent long-term antigen 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 $\pm$ 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.