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Long-Term Exposure to House Dust Mite Leads to the Suppression of Allergic Airway Disease Despite Persistent Lung Inflammation

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Key Words
Airway eosinophilia · Airway hyperreactivity · Chronic allergen challenge · House dust mite · Macrophages

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 nonreactivity 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). The severity of AAD was compared across all stages of the model via both immunological and pulmonary parameters. ***Results:*** Short- and intermediate-term HDM exposure stimulated the development of AAD that included eosinophilia in the bronchoalveolar lavage fluid (BALF), pronounced airway hyperreactivity (AHR) and evidence of lung inflammation. Long-term HDM exposure promoted the suppression of AAD, with a loss of BALF 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-positive alveolar macrophages at the site of inflammation. ***Conclusions:*** This model recapitulates the key features of human asthma and may facilitate investigation into the mechanisms that promote immunological tolerance against clinically relevant aeroallergens.

Introduction

Allergic asthma is a chronic and debilitating disorder of the airways that impacts nearly 300 million people worldwide [1]. Unfortunately, current pharmacological therapies for asthma do not specifically alter the underlying immunopathology that contributes to the disease and have therefore had only marginal effects in reducing the overall rate

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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 a lack of immunological tolerance against inhaled, environmental antigens (Ags), that results in uncontrolled Th2 activation, pulmonary eosinophilia and airway hyperreactivity (AHR) [2]. We [3] and other groups [4–7] have demonstrated that long-term, continuous exposure to an allergen can result in the development of tolerance and the ultimate resolution of allergic airway disease (AAD) in a variety of murine models. Although previous long-term investigations into 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], suggesting 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 [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 the development of AAD and long-term HDM exposure promoted the suppression of disease, demonstrated 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 (Tregs) and a transition of alveolar macrophages (AM) to an IL-10-positive 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 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, Me., USA) and then conventionally housed in plastic cages with corncob bedding. In addition, B6(Cg)-*Il10*$^{tm1.1Karp}$/J ('Vert-x') mice (a gift from Christopher Karp) were bred in the Center for Comparative Medicine at the University of Connecticut Health Center (UCHC), and the 6- to 8-week-old mice of both male and female sexes were used. Data for both sexes were combined because no differences were observed in disease phenotype between the 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 UCHC (protocol No. 100331–1114).

HDM Exposure Protocol

Mice were lightly anesthetized with vaporous isoflurane and intranasally (i.n.) instilled with droplets containing 25 μg of lyophilized HDM extract (an equal mixture of *D. pteronyssinus* and *D. farinae*, Greer Laboratories, Lenoir, N.C., USA) solubilized in 50 μl phosphate-buffered saline (PBS). This dose has previously been associated with peak inflammation in response to i.n. HDM and is 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]. The total lipopolysaccharide (LPS) content in the HDM extract was 1,250 U of endotoxin/mg of HDM (i.e. approx. 0.1 μg LPS/mg HDM [15]; 0.01% contamination).

HDM was administered for 5 consecutive days, followed by 2 days of rest, for up to 11 consecutive weeks (fig. 1). Control groups received equal volumes of i.n. PBS in a time-matched manner. Mice were sacrificed after short-term (2 weeks), intermediate-term (5 weeks) or long-term (11 weeks) Ag exposure. All mice were sacrificed 72 h after the final Ag challenge.

Bronchoalveolar Lavage Fluid and Tissue Analysis

At sacrifice, bronchoalveolar lavage fluid (BALF), hilar lymph node (HLN) and lungs of each animal were harvested and processed for the 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

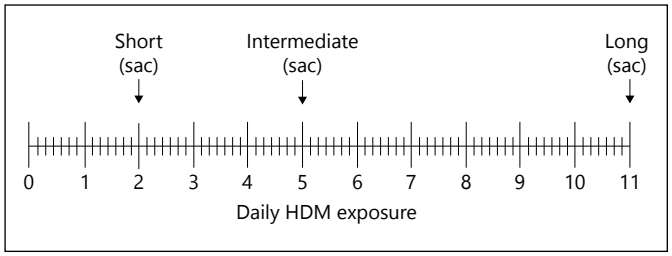

Fig. 1. Continuous model of HDM-induced AAD. C57BL/6 mice were lightly anesthetized with isoflurane and administered 25 μg of lyophilized HDM extract in 50 μl PBS i.n. for up to 11 weeks as described in Materials and Methods. Mice were sacrificed (sac) after either short-term (2 weeks) exposure, intermediate-term (5 weeks) exposure, or long-term (11 weeks) exposure. Control groups received i.n. PBS in a similar manner.

exclusion as a measure of viability. Cytospin preparations of BALF 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 BALF, HLN and lung tissue were analyzed via flow cytometry using the following monoclonal antibodies: anti-F4/80 (BM8.1; Tonbo Biosciences, San Diego, Calif., USA), anti-CD11b (M1/70; eBioscience, San Diego, Calif., USA), 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% 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, Grand Island, N.Y., USA) 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, N.J., USA) and analyzed with FlowJo (Tree Star Software, Ashland, Oreg., USA).

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 UCHC 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 0–3 as similarly described [7]. For inflammation scores, a value of '0' was assigned when no inflammation was detectable, '1' for mild peribronchiolar/perivascular cuffing with inflammatory cells, '2' for significant peribronchiolar/perivascular clustering and '3' for significant clustering and airway remodeling. For mucus scores, a value of '0' was assigned when no mucus was present, '1' for occasional and punctate mucus staining in the airways, '2' for the presence of ring-like mucus structures in <10% of the airways and '3' for the presence of ring-like mucus structures in >10% of the airways.

Determination of Serum Immunoglobulin Levels

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

For the detection of serum HDM-specific IgE, 96-well, Nunc MaxiSorp, flat-bottom plates (Thermo Scientific, Waltham, Mass., USA) were coated with 10 μg/ml of HDM extract in sodium bicarbonate buffer (pH 9.5) for 16 h at 4°C. Following washing, the plates were blocked for 1 h at 37°C with BD OptEIA assay diluent (BD Biosciences, San Diego, Calif., USA). After another washing, serum samples were added as 2-fold serial dilutions (range: 1/20–1/2,560) and allowed to incubate for 1.5 h at room temperature. The plates were washed 8 times, after which, biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech, Birmingham, Ala., USA) was applied, followed by streptavidin-HRP (BD Biosciences).

The detection of serum HDM-specific IgG_1 was performed as similarly described [12]. Plates were coated with 2 μg/ml of HDM extract in sodium bicarbonate buffer for 16 h at 4°C. Following washing, they were blocked for 1 h at room temperature with BD OptEIA assay diluent. After another washing, the serum samples were added as 10-fold serial dilutions (range: 1/20–1/200,000,000) and were allowed to incubate for 1.5 h at room temperature. The plates were washed 8 times, after which, biotin-SP-conjugated goat anti-mouse IgG Fcγ subclass 1-specific antibody (Jackson ImmunoResearch, West Grove, Pa., USA) was applied, followed by streptavidin-HRP (BD Biosciences).

For both assays, development was with 3,3′,5,5′-tetramethylbenzidine, and the reaction was stopped with an equal volume of 1 M of phosphoric acid. Dual absorbance at 450 and 570 nm was measured with a model 480 microplate reader from Bio-Rad (Hercules, Calif., USA).

Quantification of Cytokines

Detection of cytokines was performed using a custom Milliplex MAP mouse cytokine magnetic bead panel (EMD Millipore, Billerica, Mass., USA). Serum samples were diluted 2-fold and BAL supernatant samples were concentrated 10-fold. Cells from lung tissue were processed into a single-cell suspension and lysed via sonication. Cells were spun at 300 *g* for 5 min at 4°C and supernatant was concentrated 10-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 AHR

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., USA). Mice were anesthetized intraperitoneally with 100 μg/kg nembutol sodium solution (Ovation Pharmaceuticals Inc., Deerfield, Ill., USA). Mice underwent tracheostomies and were mechanically ventilated using the flexiVent system (SCIREQ, Montreal, Calif., USA). To paralyze the respiratory muscles prior to the onset of methacholine exposure, the animals were intraper-

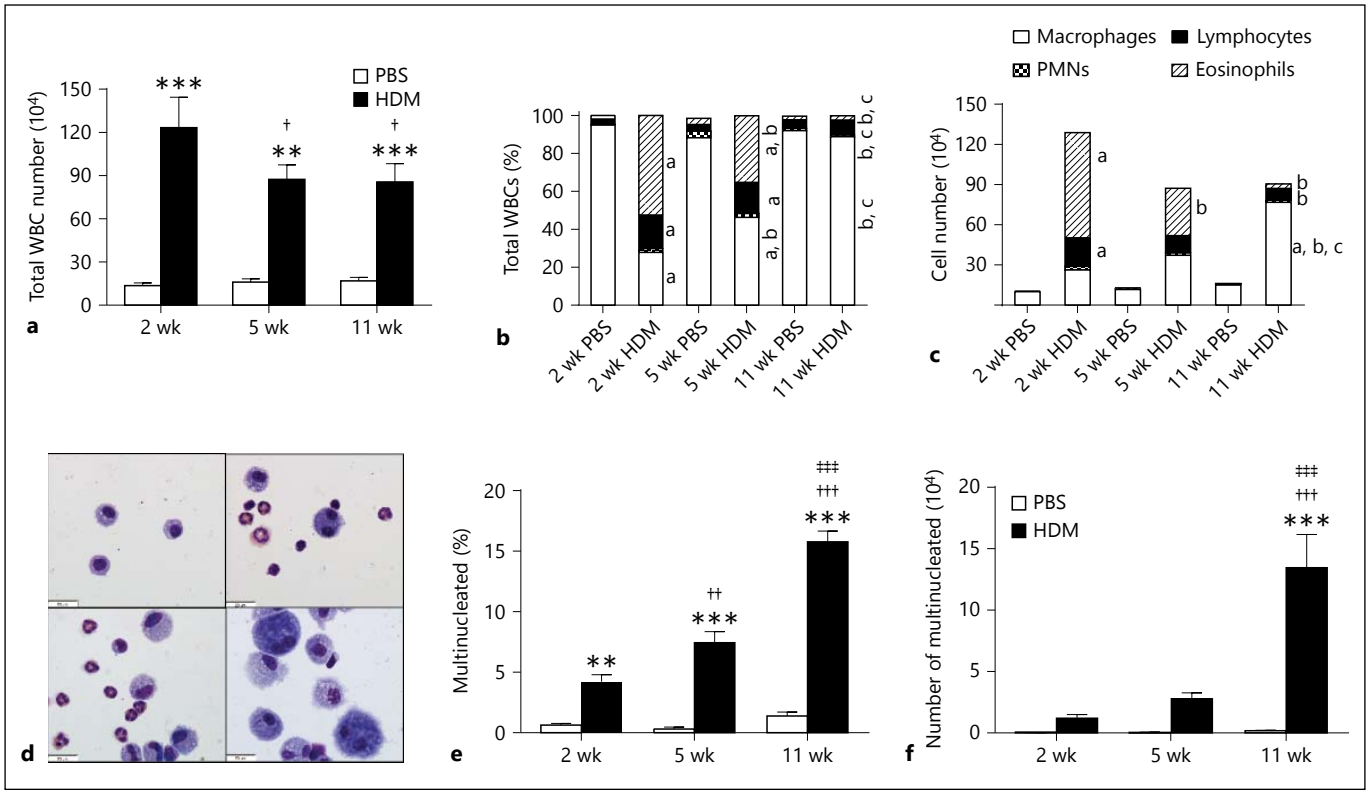

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Fig. 2. BALF eosinophilia peaks with short-term HDM exposure and resolves with long-term HDM exposure. Female C57BL/6 mice were exposed i.n. to PBS or HDM extract for up to 11 weeks (wk) (see fig. 1). **a** At sacrifice, total BALF leukocytes were harvested and manually counted on a hemocytometer. **b–d** Cytocentrifuged preparations were stained with May-Grünwald and Giemsa and differential analysis was manually performed. **d** Short-term PBS control (upper left panel), short-term HDM (upper right), intermediate-term HDM (lower left) and long-term HDM (lower right). Scale bar = 20 μm. ×60. **e**, **f** Further analysis was performed on macrophage populations from (**d**) and the percentage (**e**) and number (**f**) of multinucleated versus mononucleated macrophages were manually determined. **e** Values 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). PMN = Polymorphonuclear neutrophil; WBC = white blood cell. **a**, **e**, **f** ** $p < 0.01$, *** $p < 0.001$ vs. time-matched PBS controls; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. short-term HDM; ‡‡‡ $p < 0.001$ vs. intermediate-term HDM. **b**, **c** [a] $p < 0.05$ vs. time-matched PBS controls; [b] $p < 0.05$ vs. short-term HDM; [c] $p < 0.05$ vs. intermediate-term HDM.

itoneally administered 500 ng/kg pancuronium bromide (Sigma-Aldrich). Airway reactivity was determined by assessing forced oscillatory mechanics every 10 s for 4 min 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 the Neuman-Keuls post hoc test. AHR and serum immunoglobulin curves were compared via area under the curve (AUC) measurements. One-way ANOVA was then performed on the AUC data with the Neuman-Keuls post hoc test. Cytokine data and histopathology scores were compared via the Kruskal-Wallis test with the Dunn 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, Calif., USA) statistical software package.

Results

Short- and Intermediate-Term HDM Exposure Resulted in Elevated Eosinophil Levels in the BALF and Long-Term Exposure Led to a Resolution of BALF Eosinophilia

In agreement with previous studies that have examined responses to acute HDM instillation using similar models [13], we noted a dramatic (10-fold) increase in total BALF leukocytes after short-term (2 weeks) administration when compared to time-matched PBS control animals (fig. 2a). Cell counts decreased significantly following intermediate-term (5 weeks) HDM administration and remained at a similar level following long-term (11 weeks) HDM exposure. However, total BALF leuko-

cytes remained elevated compared to time-matched control animals following both intermediate- and long-term HDM exposure, indicating persistent inflammation.

As was expected, mice exposed to PBS at all of the time points harbored only a few (<3%) eosinophils and neutrophils in the BALF (fig. 2b). HDM-exposed mice did not show any elevation in neutrophil levels compared to the time-matched control animals, but short- and intermediate-term exposure to HDM resulted in a dramatic increase in eosinophils compared to the controls ($p < 0.001$). The total number of eosinophils was significantly elevated after short-term HDM exposure (fig. 2 a,c). It was also elevated after intermediate-term HDM exposure, but did not reach statistical significance due to the high variation in eosinophil numbers at this time point ($35 \pm 8 \times 10^4$). Despite the continued elevation of total leukocyte counts, long-term HDM exposure led to a reduction in BAL eosinophil levels that matched the control animals (fig. 2b*)*. In addition, BALF 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 2a, c) after short-term exposure but was greatly decreased following long-term HDM exposure. Together, these findings suggest that long-term HDM exposure promotes the suppression of allergic inflammation in spite of persistent BALF total leukocyte counts.

In the PBS control mice, 95% of the BALF cells were macrophages (fig. 2b). Macrophage frequency dropped to 28 ± 4% in short-term HDM-exposed 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 the time-matched control mice. However, absolute numbers of macrophages in long-term HDM-exposed mice increased 5-fold compared to the time-matched control mice and 2- to 3-fold compared to the short- and intermediate-term HDM-exposed mice (fig. 2c). Moreover, on histology, there was a dramatic alteration in the appearance of the airway macrophages over the course of the disease (fig. 2d). The macrophages from the PBS control mice appeared small, round and inactive (fig. 2d, upper left) at all time points, but those from the short- and intermediate-term HDM-exposed mice (fig. 2d, upper right and lower left, respectively) were elongated and vacuolated, and began to show evidence of multinucleation (fig. 2d, e). The macrophages from the long-term HDM-exposed mice had increased dramatically in size compared to controls and short- and intermediate-term HDM-exposed macrophages (fig. 2d, lower right). In addition, there was a substantial increase in the frequency and number (fig. 2e and f, respectively) of multinucleated macrophages at this time point compared to those found in controls and short- and intermediate-term HDM-exposed animals, thus indicating that macrophage phenotype was altered over the course of the 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, compared to the time-matched PBS controls, significant elevations in HDM-specific IgE were observed only in the 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 the long-term HDM-exposed mice were also significantly elevated compared to in the short- and intermediate-term HDM-exposed mice, suggesting ongoing systemic allergic inflammation. Of note, the total serum IgE titers in the short-term HDM-exposed mice were found to be elevated above the levels in the time-matched PBS controls, and did not change significantly following intermediate- or long-term HDM exposure (data not shown). HDM-specific IgG_1 levels followed a similar trend to 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 the HDM-exposed mice. Levels of IL-4 in the BALF and lung tissue of short-term HDM-exposed mice were significantly increased compared to the controls, but decreased following long-term HDM exposure (fig. 4). IL-5, which did not appear to be greatly elevated in the BALF of the 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 the disease, demonstrating additional evidence of ongoing, systemic inflammation. In addition, the proregulatory 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 BALF and detected 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 [18]. Local (BALF and

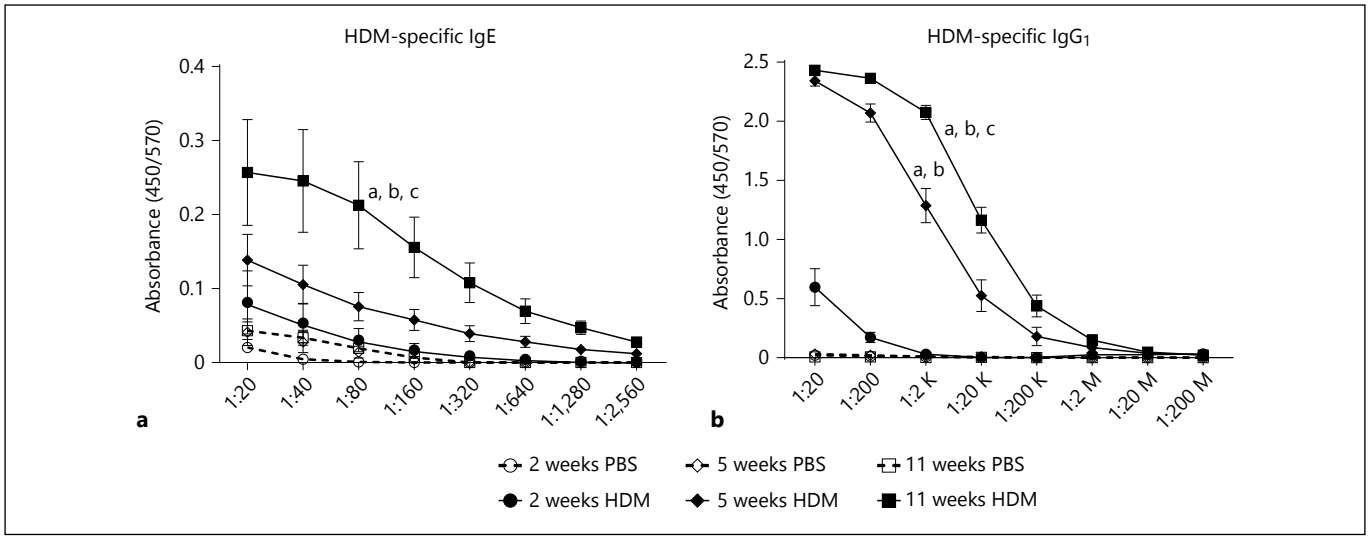


Fig. 3. HDM-specific IgE (**a**) and IgG_1 (**b**) gradually increase over the course of HDM exposure. At sacrifice, serum was collected from both naïve and HDM-exposed animals. HDM-specific IgE and IgG_1 levels were determined via ELISA. Dual absorbance at 450 and 570 nm was calculated over a range of dilutions. Data represent mean ± SEM values; n = 6–8 mice per group. For statistical purposes, group comparisons were based on AUC measurements. [a] $p < 0.01$ vs. AUC of time-matched PBS controls; [b] $p < 0.01$ vs. AUC of short-term HDM; [c] $p < 0.01$ vs. AUC of intermediate-term HDM. K = 1,000; M = 1,000,000.

lung tissue) INFγ levels approximated or fell below the limit of detection in the HDM-exposed mice at all time points (fig. 4). 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 when compared to the control mice or the short-term HDM-exposed 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-Exposed 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 weeks) HDM exposure in the form of small, inflammatory pockets (fig. 5b, short arrow) and increased following intermediate-term (5 weeks) 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 BALF eosinophilia was attenuated in the long-term HDM-exposed mice (fig. 2c, d), diffuse peribronchiolar and perivascular inflammation in the lung tissue peaked at this time point (fig. 5d, short arrow). Semiquantitative analysis supported an increase in inflammation compared to the time-matched PBS controls (fig. 5i). The composition of this inflammation, however, changed relative to the short- and intermediate-term HDM-exposed mice, such that it was predominantly comprised of macrophages and lymphocytes with only a few eosinophils present. No further increase in smooth-muscle hypertrophy was noted in the long-term HDM-exposed 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 the long term HDM-exposed mice when compared to other stages of the model (data not shown).

In the controls, there was no evidence of increased mucus production in the smaller airways (fig. 5e). Animals exposed to HDM for short- and intermediate-term periods demonstrated increased mucus production relative to controls via the positive PAS staining of the goblet cells in the 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 the airways of the long-term HDM-exposed mice when tissue samples were blindly scored [fig. 5h (arrows), j]. The resolution of airway eosinophilia reduced the number of Th2 cytokines in the

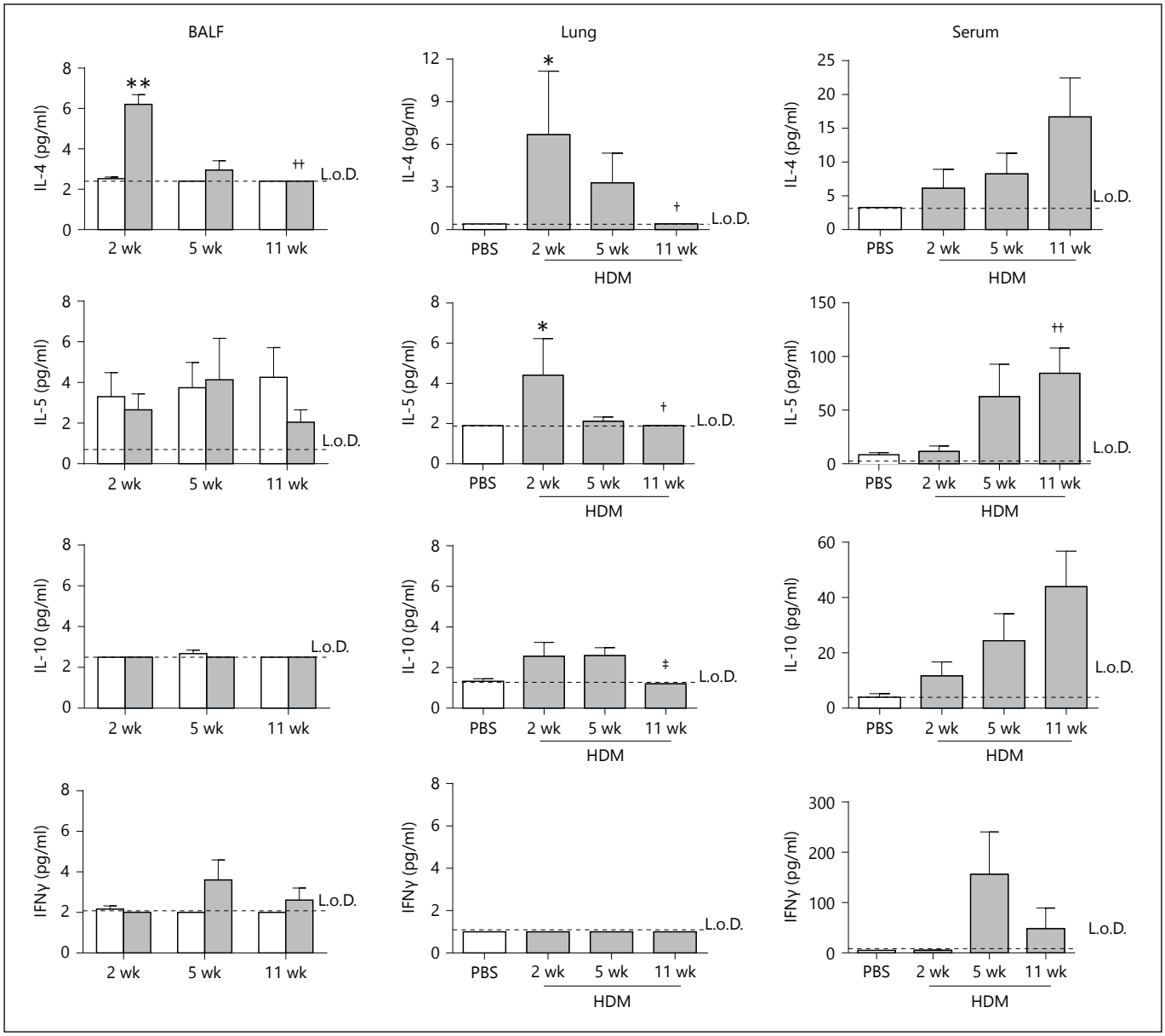


Fig. 4. Th2 cytokines peak in the lung compartments following short-term HDM exposure. At sacrifice, BALF supernatant was collected from PBS-exposed (unshaded bars) and HDM-exposed (shaded bars) C57BL/6 females and was concentrated 10-fold. Lung tissue homogenates and serum were collected from PBS (short-term only) and HDM animals. Cytokine levels were determined via multiplex analysis. Data represent mean ± SEM values; n = 5 per group (BALF), 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.

lung compartments and the attenuated airway mucus production suggested that long-term exposure to HDM led to the suppression of allergic inflammation. Nevertheless, elevated BALF leukocyte counts and peribronchiolar/perivascular inflammation persisted at this stage of the model.

Lung Resistance Was Elevated by Intermediate-Term HDM Exposure and Ameliorated by Long-Term HDM Exposure

In order to determine whether the resolution of BALF eosinophilia and attenuation of mucus production with long-term HDM exposure correlated with improved

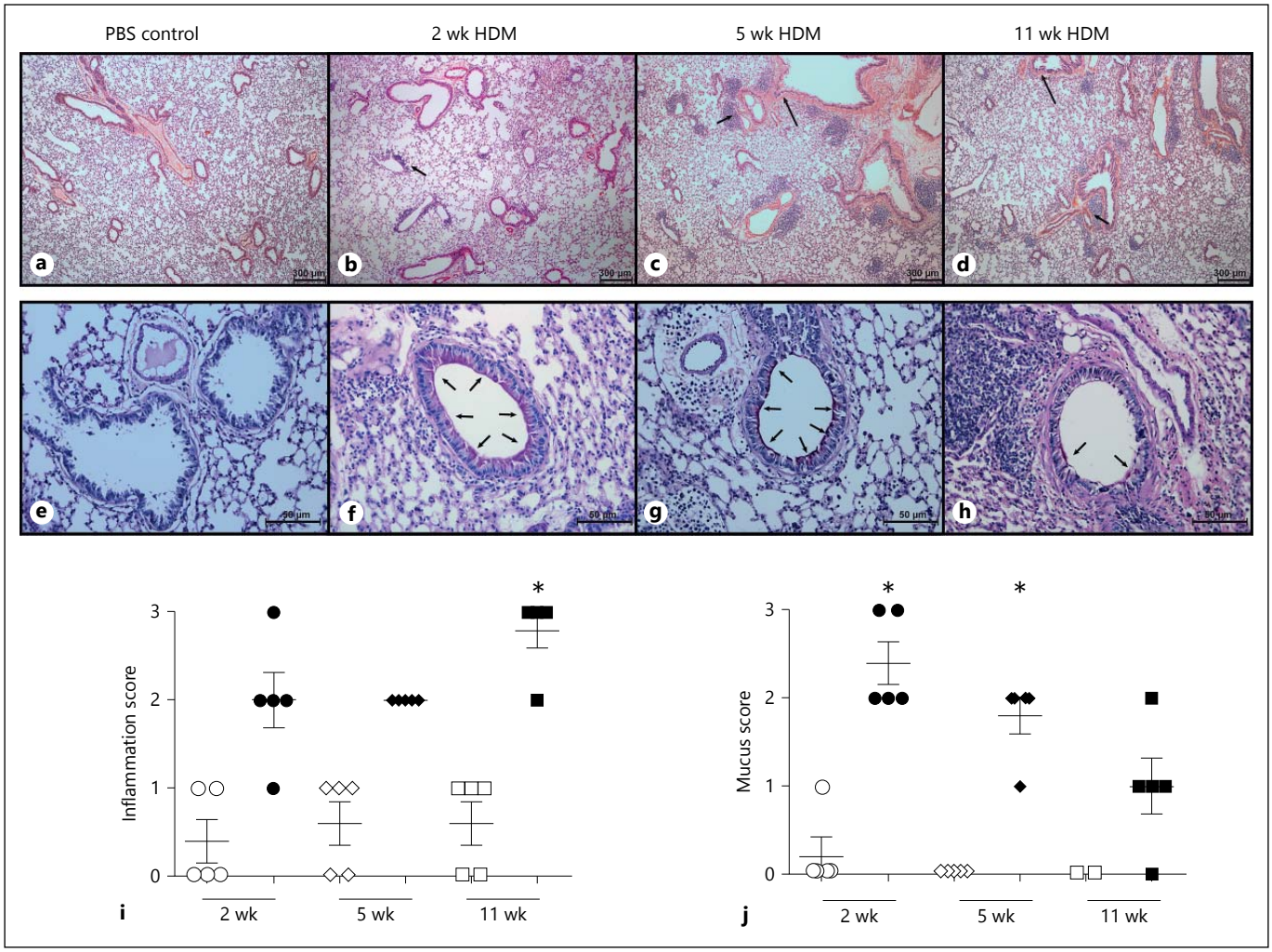


Fig. 5. HDM-exposed mice demonstrated persistent perivascular/peribronchiolar inflammation. Formalin-fixed lungs were processed in a standard manner and stained. **a–d** Short arrows highlight examples of perivascular/peribronchiolar inflammation. Long arrows highlight examples of smooth muscle hypertrophy. Scale bar = 300 μm. H&E. ×4. **e–h** Arrows highlight mucus production by airway goblet cells. Scale bar = 50 μm. PAS. ×20. Inflammation scores (**i**) and mucus scores (**j**) were determined in a blinded fashion on a severity scale from 0–3 for PBS controls (open shapes) and HDM (closed shapes) mice. Data represent mean ± SEM values; n = 5 per group. wk = Weeks. * $p < 0.05$ vs. time-matched PBS controls.

airway dynamics, AHR was assessed in response to methacholine challenge in both the PBS control and HDM-exposed mice. Baseline Rrs measurements in the PBS mice at 2, 5 and 11 weeks were virtually identical and were thus pooled to obtain a single curve representing all PBS-exposed animals. As expected, Rrs measurements in the PBS-exposed mice and all groups of the 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 1.25–100 mg/ml. Compared to PBS mice, short-term HDM-exposed 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 HDM-exposed mice demonstrated a significant elevation in Rrs compared to PBS controls. The Rrs for the intermediate-term HDM-exposed mice was also significantly elevated compared to the short-term HDM-exposed mice, suggesting that intermediate-term HDM exposure led to peak airway dysfunction. The increase in AHR at 5 weeks was completely diminished with long-term HDM administration, such that the Rrs curves in the PBS- and long-term HDM-exposed mice

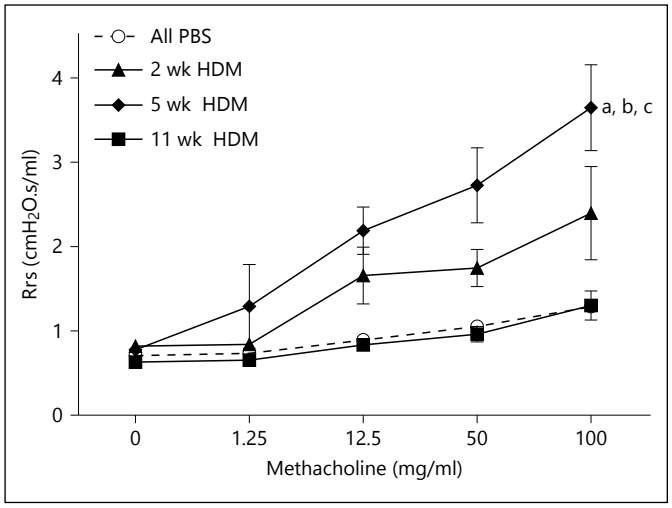


Fig. 6. AHR peaked following intermediate-term HDM exposure but was suppressed with long-term HDM exposure. C57BL/6 females underwent tracheostomy and were mechanically ventilated prior to methacholine exposure. Changes in Rrs responses to increasing doses of aerosolized methacholine were determined using the flexiVent system (SCIREQ). Since no differences were observed within AHR measurements for PBS control animals at 2, 5 or 11 weeks (wk), AHR data from all PBS control mice were pooled to generate a single curve. Data represent mean ± SEM values; n = 4–5 per group (HDM), n = 8 (all PBS). For statistical purposes, group comparisons were based on 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.

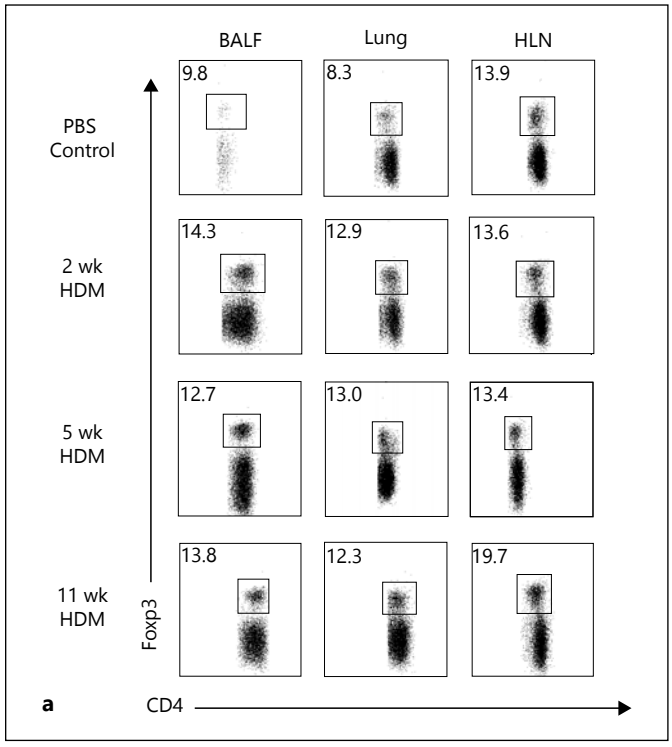
did not statistically differ from one another. The correlation between the resolution of airway eosinophilia (fig. 2b) and AHR provided additional evidence that long-term exposure to HDM promotes suppression of AAD despite persistent perivascular/peribronchiolar lung inflammation.

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 Ags occur at the site of inflammation [17, 19]. As a result, we examined percentages and total numbers of Foxp3+ Tregs in the lung compartments (BALF, HLN and lung tissue) over an 11-week course of HDM exposure. When compared with time-matched PBS control mice, short-term HDM-exposed mice demonstrated a significant increase in the frequency of Foxp3+ T cells relative to total CD4+ T cells in the BALF 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 BALF and lung tissue was not further elevated in the long-term HDM-exposed mice when compared to short- or intermediate-term HDM-exposed animals. Total numbers of Foxp3+ Tregs in the BALF and lung tissue were significantly increased compared to 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 the long-term HDM-exposed mice relative to the short- or intermediate-term HDM-exposed mice (fig. 7c).

Foxp3+ Treg frequency among total CD4+ T cells in the HLN was approximately 14 ± 2% in the PBS control group and was not significantly elevated with short- or intermediate-term HDM exposure (fig. 7a, b). However, long-term HDM-exposed mice demonstrated significant increases in HLN Foxp3+ Treg frequency (20 ± 2%) when compared to time-matched PBS controls and short- and intermediate-term HDM-exposed 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 the resolution of eosinophilia in the airways (fig. 2b, c). Of note, no major differences were observed in the 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 the 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. 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 the status of M2 AMs (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 PBS to IL-10-GFP reporter (Vert-x) mice for both a short- (2 weeks) and long-term (11 weeks) period as described in figure 1. While neither PBS nor short-term HDM administration increased IL-10 production by AMs in the BALF or lung tissue compared to negative control animals, the AMs from long-term HDM mice demonstrated a rightward shift in GFP expression indicative of elevated IL-10 pro-



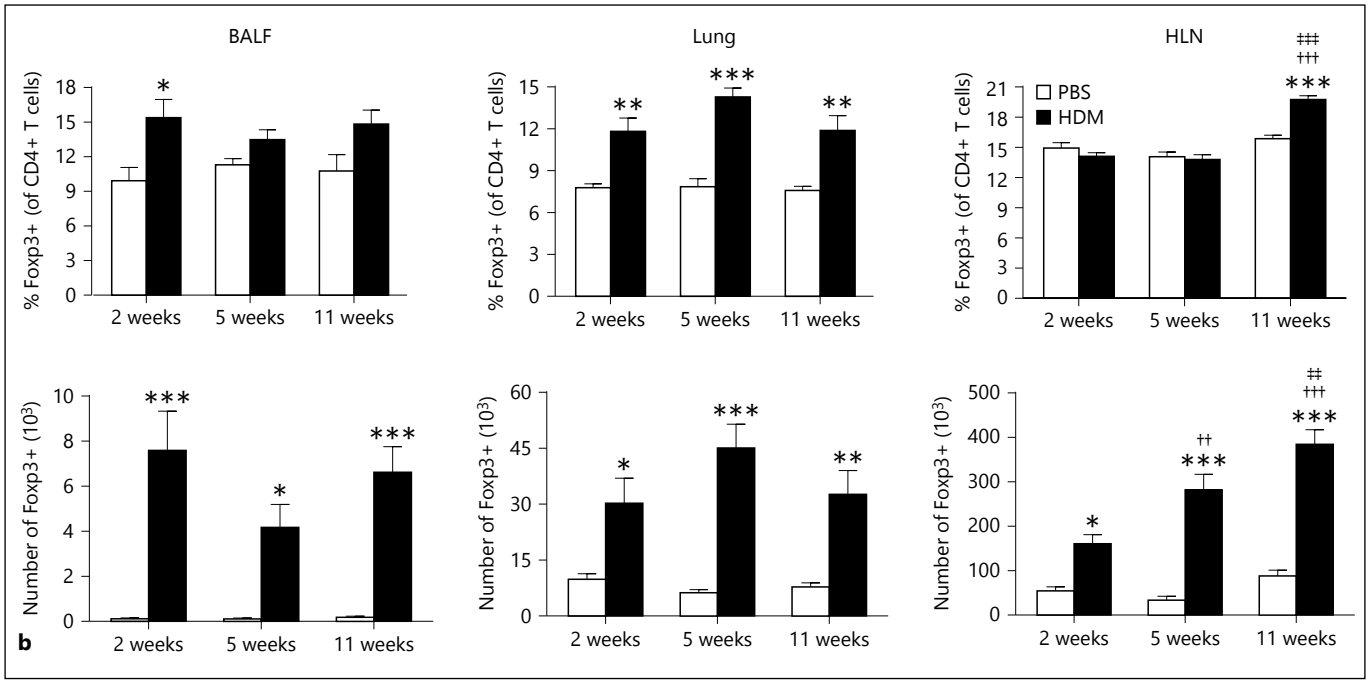


Fig. 7. Foxp3+ Treg numbers increase steadily in the HLN over the course of HDM exposure and correlate with suppression of AAD. **a** Representative flow cytometry dot plots from BALF, lung tissue and HLN of PBS controls (short-term) and HDM-exposed C57BL/6 females. Cells were gated on CD3+CD4+ T lymphocytes and Foxp3 expression was examined. **b** Total percentages and numbers of Foxp3+ Tregs of all CD4+ T cells in PBS controls or HDM-exposed mice. Data represent mean ± SEM values; n = 10–13 per group (PBS controls; 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.

duction (fig. 8b). This shift represented a significant increase in mean fluorescence intensity (MFI) of IL-10-GFP in the long-term HDM-exposed mice relative to controls and short-term HDM-exposed animals in both the BALF and lung tissue (fig. 8c). The frequency of IL-10-positive AMs in short-term HDM-exposed mice was not increased to levels greater than those observed in controls in the BALF and lung tissue (fig. 8d). However, the frequency of IL-10-positive AMs was significantly increased in the long-term HDM-exposed mice compared

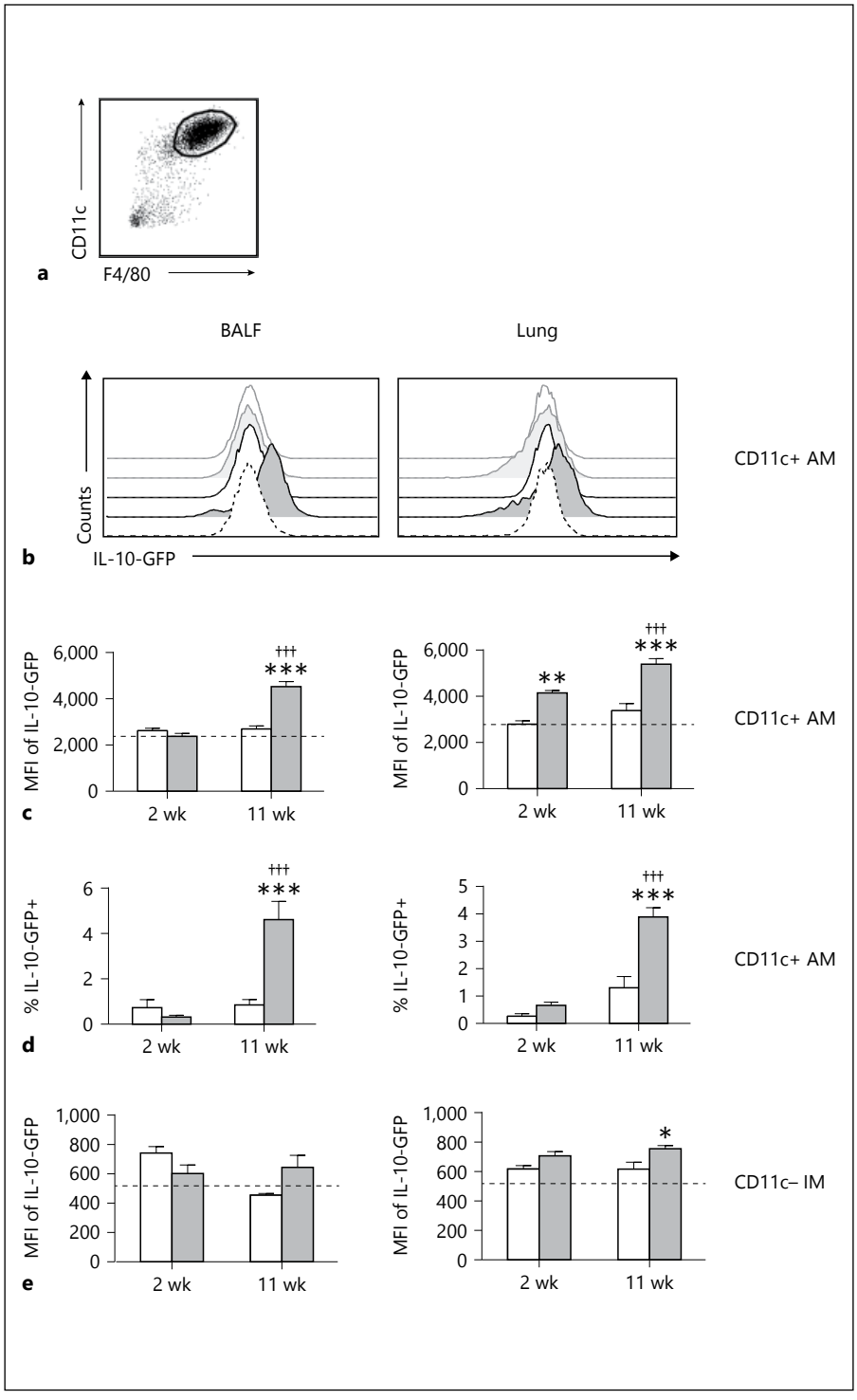


Fig. 8. AMs assume an IL-10-positive phenotype in the BALF and lung tissue following long-term HDM exposure. Male and female Vert-x mice were subjected to short- or long-term PBS or HDM exposure (see fig. 1). **a** Sample FACS plot demonstrating gating for F4/80+CD11c+ AMs (encircled) among the population of total BALF leukocytes in a 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-negative AMs from a wild-type 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-negative AMs from a wild-type mouse). **d** Frequency of IL-10 GFP-positive 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-negative IMs from a wild-type mouse. Data represent mean ± SEM values (combined for male and females); n = 5–6 per group (representative of 3 independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. time-matched PBS control; ††† $p < 0.001$ vs. short-term HDM. **e** 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 weeks HDM administration (fig. 2d).

to controls and short-term HDM-exposed animals. Of note, no differences in frequency of IL-10-positive macrophages were observed between PBS animals at either time point, demonstrating that the induction of such macrophages was specific to HDM administration. Quite interestingly, with long-term HDM exposure, the IL-10-positive macrophages were large in size and highly granular (data not shown), thus correlating with the appearance of big, densely staining macrophages in the BALF after 11 weeks of HDM administration (fig. 2d).

Discussion

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

Our laboratory has previously demonstrated that continuous aerosol administration of OVA leads to a biphasic response in which short-term exposure promotes AAD that resolves following long-term exposure [3]. Long-term HDM administration showed a similar pattern to the long-term OVA model, i.e. suppression of AAD with diminished BALF eosinophilia and mucus production and the resolution of AHR upon methacholine challenge. In support of our findings, others have also observed an attenuation of BALF 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], which are indicators of progressive AAD rather than attenuated AAD. The reason for this discrepancy between models is not entirely known, but one might speculate that it is 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 intratracheally exposed to the fungal allergen *Aspergillus fumigatus* demonstrated a neutrophil predominance (compared with C57BL/6 mice) due to augmented TNFα production by dendritic cells and macrophages [23]. TNFα has also 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 a limited dependency of allergen dose on pathology, cytokine responses and AHR in short-term HDM-exposed mice [25], dose effects in long-term HDM exposure have not been well characterized and require appropriate follow-up studies.

Although the effects of long-term OVA exposure on the suppression of methacholine-induced AHR are well established, this study is the first to relate the resolution of eosinophilia with a 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 plays a causal role in this response [26]. One study showed that long-term HDM exposure, i.e. 20 weeks, did not result in the resolution of AHR [21], but HDM was administered only 3 times per week as opposed to 5 times. Thus, continuous Ag instillation (a hallmark of both our OVA and HDM protocols) may be required for tolerance induction and the subsequent suppression of AAD. In addition, a causative role between mucus overproduction and the development of AHR has been described [27]. Relative to short- 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 that we observed took place with long-term HDM exposure.

Despite the relative reduction in local inflammatory responses observed in long-term HDM-exposed mice when compared to short-term exposure, the former group demonstrated higher systemic levels of HDM-specific IgE and Th2 cytokines. Persistent (or even increased) systemic IgE responses with long-term, inhalational Ag exposure has been previously demonstrated in our laboratory [28] and by other groups [7]. Moreover, our previous findings indicate that mice that developed tolerance with long-term OVA exposure had greater subcutaneous late-phase responses after OVA footpad injection than short-term OVA-exposed mice [29]. Thus, it appears that the development of tolerance to inhaled allergens with long-term exposure is a localized response that is not dependent upon the suppression of systemic inflammation. Nevertheless, trending increases in IL-4 and IL-5 in the serum of long-term HDM-exposed mice were 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-exposed mice relative to short- and intermediate-term HDM-exposed mice. Successful courses of allergen-specific immunotherapy

(the only etiological therapy for the treatment of asthma and allergy) is commonly associated with increases in IgG_1 (the murine equivalent of IgG_4 in humans) in both mice [30] and humans [31]. 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 Th cells shift from a predominantly Th2 to a Th1 phenotype [32]. Although Th2 cytokines appeared to decrease locally following long-term HDM exposure, we did not observe any evidence for such a shift in our model. INFγ levels remained at or below the limit of detection in local (BALF and lung tissue) compartments. Although INFγ levels appeared to be elevated in the serum with intermediate- and long-term HDM exposure, they were associated with extremely large variations within groups. Thus, these data suggest that a switch from a Th2 to a Th1 phenotype is not likely to explain the 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 weeks of exposure [3]. A longer time course was needed for HDM, with the peak AAD seen after 5 weeks of exposure and the resolution of the AAD responses requiring 11 weeks. Moreover, long-term OVA administration is marked by full resolution of perivascular and peribronchiolar inflammation in the lung tissue, a decrease in total BALF leukocyte counts to baseline levels and little evidence of airway remodeling [3, 33]. In contrast, the mice exposed to long-term HDM had persistent inflammation in their BALF and lung tissue with some evidence of airway remodeling (in the form of smooth muscle hypertrophy). While smooth muscle hypertrophy was qualitatively increased in the long-term HDM-exposed mice relative to the short-term HDM-exposed mice, very little difference was noted when compared to airway smooth muscle in intermediate-term HDM-exposed mice. These findings are supported by previous work which demonstrated that airway smooth muscle mass increased with 5 weeks of HDM exposure but had not enlarged further with 10 weeks of HDM exposure [34]. However, in our model, the inflammatory composition in the long-term HDM-exposed mice changed from eosinophilic (2 and 5 weeks) to mononuclear (11 weeks) 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. Toll-like receptor 4 signaling from contaminating LPS in HDM extract has been clearly confirmed to contribute to the development of HDM allergy, particularly with regard to mucosal sensitization and the initiation of Th2 polarization [36]. Yet, despite the fact that mice were continuously exposed to LPS within this model, neutrophils (a key responder in LPS-mediated inflammation) were 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 BALF for some hours following the 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 the later stages of this model.

We also demonstrated previously that chronic inhalation of LPS in an aerosol solution is not required for development of immune tolerance to long-term OVA exposure; this is particularly noteworthy because our OVA and HDM extracts had similar LPS contents [38]. 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, Toll-like receptors or C-type lectin receptors 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 the apparent development of airway tolerance to HDM. Persistence of inflammation with long-term HDM exposure could represent ongoing, noneosinophilic 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-exposed animals. Thus, these long-term HDM-exposed mice appeared to have regained their respiratory tolerance to HDM, which is 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+ Tregs. 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]. The level of Foxp3+ Tregs was elevated in the HLN specifically with long-term exposure, but not with short- 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 BALF or lung tissue) demonstrated a 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 the 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 BALF 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, which suggests 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 were not characterized further [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 the BALF 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 Toll-like receptor 4 signaling through LPS exposure may stimulate this response as a feedback mechanism for curtailing inflammation [53]. However, we did not observe 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 but not in interstitial macrophages (IMs). This observation directly contrasts with previous evidence suggesting that IMs and not AMs assume an IL-10-positive phenotype upon exposure to an allergen in the presence of LPS [54]. Intriguingly, studies have demonstrated that IL-10-positive macrophages are critical for the maintenance of Foxp3 expression in mucosal Tregs [55]. Thus, it is possible that IL-10-positive AMs from long-term HDM-exposed 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 BALF 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 the capacity of AMs to express IL-10 [59]. However, it has yet to be established whether the IL-10-positive AMs induced by chronic HDM exposure are indeed active contributors to disease suppression or simply markers of a tolerogenic lung environment.

In summary, our study provides the first evidence that long-term HDM exposure leads to the suppression of the AAD associated with short- and intermediate-term HDM exposure; this suppression includes the 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. Fundamental differences from the OVA model were noted with long-term HDM instillation, including persistent mononucle-

ar inflammation in the peribronchiolar/perivascular regions of the lung despite abrogation of AHR as well as the formation of IL-10-positive AMs. We believe that the 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.

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Disclosure Statement

The authors declare no conflicts of interest.

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