

Eosinophils Are Dispensable for Allergic Remodeling and Immunity in a Model of House Dust Mite–induced Airway Disease

Ramzi Fattouh¹, Amal Al-Garawi¹, Marcia Fattouh¹, Katherine Arias¹, Tina D. Walker¹, Susanna Goncharova¹, Anthony J. Coyle², Alison A. Humbles², and Manel Jordana¹

¹Division of Respiratory Diseases and Allergy, Centre for Gene Therapeutics and Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada; and ²Department of Inflammation and Autoimmunity, MedImmune LLC, Gaithersburg, Maryland

Rationale: Current thinking accredits eosinophils with preeminent contributions to allergic airway responses, including a major role in the development of airway remodeling, a process thought to significantly contribute to airway dysfunction. However, direct evidence in support of this notion is limited and often controversial. **Objectives:** We elucidated the requirement for eosinophils in the generation of allergic sensitization, airway inflammation, and remodeling in a model involving chronic respiratory exposure to house dust mite (HDM).

Methods: We used three methods to selectively eliminate eosinophils, a depleting antibody (anti-CCR3), and two strains of eosinophil-deficient mice (Δ dblGATA and the transgenic line *PHIL*).

Measurements and Main Results: Anti-CCR3 treatment markedly reduced pulmonary eosinophilia ($> 80\%$) over the course of HDM exposure but had no effect on the remaining inflammatory response, the extent of lung Th2 cells, or the development of remodeling-associated changes, including subepithelial collagen deposition and smooth muscle thickening. In addition, we observed that, despite the absence of eosinophils, HDM-exposed GATA mice mounted robust airway and lung inflammation and hyperresponsiveness and showed a remodeling response equivalent to that observed in wild-type mice. Moreover, these mice had similar serum HDM-specific IgE levels and Th2-associated splenocyte cytokine production as HDM-exposed wild-type control mice. Similar observations were made in *PHIL* eosinophil-deficient mice subjected to chronic HDM exposure, although slight decreases in airway mononuclear cells, but not lung Th2 cells, and remodeling were noted.

Conclusions: Collectively, these data demonstrate that, at variance with the prevailing paradigm, eosinophils play negligible roles in the generation of HDM-induced allergic immunity and airway remodeling.

Keywords: airway remodeling; house dust mite; allergic asthma; eosinophils; mouse

Allergic asthma is a chronic immune-inflammatory driven disease that involves the development of various alterations to the structure of the airway walls. These alterations, collectively termed “airway remodeling,” include: (1) goblet cell hyperplasia and increased epithelial cell turnover and mucus production; (2) thickening of the extracellular matrix below the epithelium

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The current thinking, based largely on experimental evidence from studies using conventional ovalbumin-based models of allergic airway disease, is that eosinophils play a prominent role in the development of airway remodeling, a characteristic feature of chronic allergic asthma.

What this Study Adds to the Field

This research contributes to our understanding of the mechanisms regulating Th2-polarized inflammation, immunity, and remodeling in chronic allergic asthma and suggests that therapeutic strategies to prevent remodeling through eosinophil depletion may have negligible effects.

due to increased deposition of matrix proteins such as collagen, fibronectin, and laminin, among others; (3) increased smooth muscle mass on account of smooth muscle cell hypertrophy and/or hyperplasia; and (4) an increase in the small blood vessel area (1, 2). Although not exempt of controversy, the evidence regarding the clinical and functional impact of remodeling indicates that at least some elements of it play a significant part (2, 3).

Various cell types, both structural and immune, have been implicated in airway remodeling. In particular, eosinophils are thought to be principally involved because they are able to produce a multitude of cytokines, growth factors, and toxic mediators that can influence the function, proliferation, and differentiation of resident structural lung cells and, thus, could drive airway remodeling. Experimental studies using interleukin (IL)-5 and eotaxin-overexpressing transgenic mice have shown that eosinophils can elicit many of the hallmark pathologies of allergic airway disease, including airway remodeling (4). However, whether eosinophils actually do contribute to the remodeled airway in other experimental systems or in patients with allergic asthma remains debatable. Additional investigations in mouse models examining the requirement for eosinophils in the development of airway remodeling indirectly by interfering with IL-5 function have yielded disparate results (5–10). Interestingly, significant reductions in subepithelial collagen deposition and smooth muscle cell proliferation were observed in sensitized and chronically challenged Δ dblGATA eosinophil-deficient mice compared with wild-type (WT) control mice (11), suggesting that eosinophils may play a central role in remodeling. A common feature of these studies is that they all used ovalbumin (OVA)-based models. This, we surmise, is important because a number of key differences, mechanistic and otherwise, are emerging between OVA-based models and models using a common environmental allergen (CEA). For example, although

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Correspondence and requests for reprints should be addressed to Manel Jordana, M.D., Ph.D., Professor, Department of Pathology and Molecular Medicine, Head, Division of Respiratory Diseases and Allergy, McMaster University, 1200 Main Street West, Hamilton, ON, L8N 3Z5, Canada, MDCL 4013. E-mail: jordanam@mcmaster.ca

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transforming growth factor (TGF)- β has been shown to be an integral mediator of remodeling in the OVA systems (12–14), TGF- β was not critically required for the development of house dust mite (HDM)-induced remodeling (15). To date, the role of eosinophils in remodeling has not been examined in an experimental system using a CEA. Thus, in this study we investigated the role of eosinophils in a chronic model of HDM-induced allergic airway remodeling and adaptive immunity. Our data conclusively show that eosinophils play a negligible role in these processes.

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

METHODS

Animals

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

Sensitization Protocol

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

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

Collection and Measurement of Specimens

Bronchoalveolar lavage (BAL) fluid, lungs, blood, and spleen were collected approximately 72 hours after the last HDM challenge. Additional details on the methods used to perform BAL are provided in the online supplement. Protocol Hema 3 stain set (Fisher Scientific, Toronto, ON, Canada) was used to stain all smears. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as eosinophils, mononuclear cells, or neutrophils. Where applicable, after BAL, the lungs were perfused through the right ventricle with 10 ml of warm Hank's balanced salt solution (HBSS), and the right lung was dissected and placed into HBSS at 4°C for lung cell isolation. The left lung was inflated with 10% formalin at a constant pressure of 20 cm H₂O and fixed in 10% formalin for 48 to 72 hours for histochemical and morphometric analysis. Peripheral blood was collected by retroorbital bleeding. Serum was obtained by centrifugation of whole blood after incubation for 30 minutes at 37°C and stored at –20°C. For peripheral blood leukocyte isolation, up to 1 ml of blood was collected in a tube containing 50 μ l of 100 mM ethylenediaminetetraacetic acid in PBS (pH 7.2–7.4). Harvested spleens were placed in sterile tubes containing sterile HBSS.

Histology and Immunohistochemistry

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

Morphometric Analysis

Details on the methods used to make these measurements are provided in the online supplement.

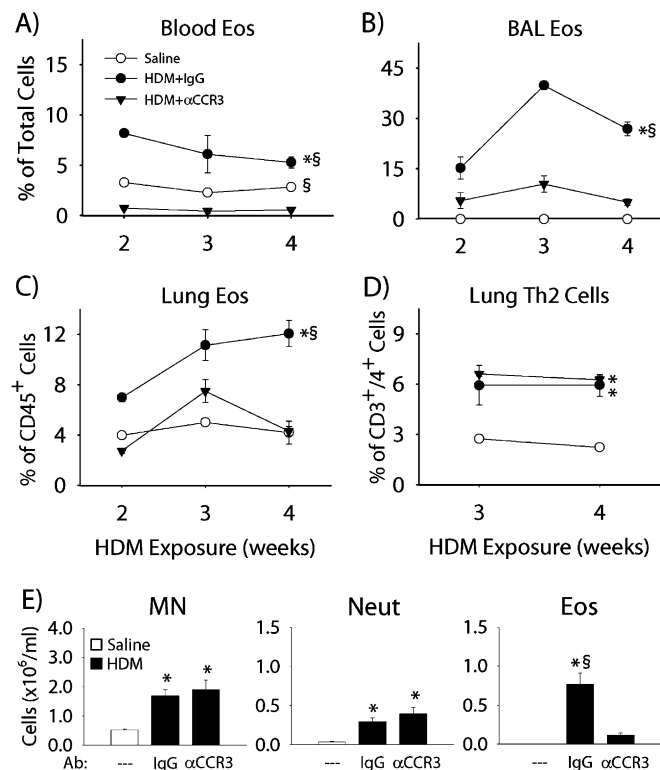


Figure 1. Systemic and local impact of anti-CCR3 (α CCR3) treatment on eosinophils and Th2 cells in house dust mite (HDM)-exposed mice. Separate groups of mice were exposed to HDM extract or saline for up to 4 weeks. HDM-exposed mice were concurrently treated on Days 3, 6, 9, 13, 16, 19, 22, and 25 with an α CCR3 eosinophil-depleting antibody (Ab) or a control IgG Ab (0.5 mg per dose). Different groups of mice were killed 24 hours after the second and third weeks of exposure or 72 hours after the last HDM or saline exposure (i.e., in the fourth week). (A) Percentage of eosinophils (Eos) in the blood and (B) the bronchoalveolar lavage (BAL) based on differential cell count analysis. (C) Percentage of Eos (CD45⁺ Gr-1^{low/int} SSC^{int/high}) and (D) Th2 cells (CD45⁺ CD3⁺ CD4⁺ T1/ST2⁺) in the lung based on flow cytometric analysis of whole lung digest using the gating strategies described in Figure E1 of the online supplement. (E) Differential cell count analysis showing the numbers of mononuclear cells (MN), neutrophils (Neut), and eosinophils in the BAL at the 4-week time point. (A–D) $n = 2$ to 3 mice per group for the 2- and 3-week time points. (A) $n = 5$ to 7 mice per group, and (B–E) $n = 6$ or 7 mice per group for the 4-week time point. Data are expressed as mean $\pm$ SEM where possible. $P < 0.05$ compared with the *saline-exposed group and §HDM-exposed α CCR3-treated group. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test on the 4-week time point only.

Peripheral Blood Leukocyte and Lung Cell Isolation

Details on the methods used to make these measurements are provided in the online supplement.

Flow Cytometric Analysis

To minimize nonspecific binding, cells were first incubated with FcBlock (anti-CD16/CD32; BD PharMingen, Mississauga, ON, Canada) in FACS buffer (0.5% BSA in PBS) for 15 minutes at 4°C. Antibodies were titrated to determine optimal concentration. Gating strategies used in the analysis of flow cytometric data are shown in Figure E1 in the online supplement. Additional details on the methods used to make these measurements are provided in the online supplement.

Splenocyte Culture

Splenocytes were isolated, resuspended in complete RPMI (RPMI supplemented with 10% FBS [Sigma], 1% L-glutamine, 1% penicillin/streptomycin [Invitrogen, Burnlington, ON, Canada], and 0.1% β -mercaptoethanol [Invitrogen]) at a concentration of 8×10^6 cells/ml and cultured in medium alone or with medium supplemented with HDM (31.25 μ g/ml) in a flat-bottom, 96-well plate (BD) in triplicate. After 5 days of culture, supernatants were harvested, and triplicates were pooled for cytokine measurements. Additional details on the methods used to make these measurements are provided in the online supplement.

Cytokine and Immunoglobulin Measurements by ELISA

Details on the methods used to make these measurements are provided in the online supplement.

Airway Responsiveness Measurements

Details on the methods used to make these measurements are provided in the online supplement.

Data Analysis

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

RESULTS

Impact of Anti-CCR3 Treatment on Eosinophil Kinetics and HDM-induced Remodeling

To directly investigate the requirement for eosinophils in the development of allergic remodeling induced by HDM exposure, we used a rat monoclonal antimouse CCR3 Ab (α CCR3) to selectively deplete eosinophils. The binding and depleting activity of this Ab have been previously shown to be specific for eosinophils (18). Moreover, α CCR3 has been successfully used to completely ablate eosinophils in acute OVA-induced models of allergic airway disease (19, 20). Preliminary studies were conducted to determine an optimal dosing regimen that would yield the greatest depletion over a 4-week course of HDM exposure without eliciting adverse effects (data not shown). Separate groups of BALB/c mice were exposed to saline or HDM intranasally, daily, 5 consecutive days per week for up to 4 weeks, and HDM-exposed groups were concurrently treated with 0.5 mg per dose of α CCR3 or a control Ab (IgG). Mice were killed after 2, 3, and 4 weeks of exposure, and eosinophil kinetics were examined in the blood, lung tissue, and BAL to track depletion throughout the study. α CCR3 treatment dramatically reduced blood eosinophils to levels that were below baseline (Figure 1A), and markedly decreased the extent of eosinophilia in the airway

and lung tissue compartments to levels that were at or near baseline (Figures 1B, 1C, and 1E). Based on calculations of area under the curves, we estimate that the extent of overall depletion was approximately 80 to 85% in the BAL and lung over the course of the experiment (*see* Table E1 in the online supplement). We further analyzed whole lung cells for the presence of Th2 (T1/ST2⁺ CD4⁺ CD3⁺) cells by flow cytometry. Importantly, α CCR3 treatment and the resulting decrease in eosinophilia had no impact on the extent of lung Th2 cells (Figure 1D). Notably, differential cell count analysis revealed that the number of mononuclear cells and neutrophils in the BAL at 4 weeks was also unaffected (Figure 1E).

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

Impact of Eosinophil Deletion on HDM-induced Inflammation, Immunity, and Remodeling in Δ dblGATA Mice

To confirm the results obtained from the α CCR3 studies and to exclude the possibility that the residual population of eosinophils in the lung and airways of α CCR3-treated mice was sufficient to drive the remodeling response, we subjected Δ dblGATA eosinophil-deficient mice to 5 weeks of HDM exposure. WT and eosinophil-deficient HDM-exposed mice developed robust airway inflammation as evidenced by significant increases in the BAL total cell number compared with corresponding saline-exposed control mice (Figure 3A). However, the increase in total cells observed in the GATA mice was significantly lower ($\sim$ 35% fewer cells) than that observed in WT animals. Further examination of the BAL showed that this difference was largely due to the absence of eosinophils, with only a minor decrease in mononuclear cells ($\sim$ 23% fewer mononuclear cells) and no change in neutrophils being observed in the GATA compared with WT HDM-exposed mice (Figures 3B and 3C and Fig. E2). Consistent with the BAL data, no differences in lung neutrophils were noted between the two HDM-exposed groups (data not shown), and no eosinophils were detected in the lungs of HDM-exposed GATA mice by flow cytometry (Figure 3D) and differential cell count analysis (data not shown). We also examined whether the absence of eosinophils had any effect on the T-cell component of the lung inflammatory infiltrate. To this end, we documented significant increases in CD69⁺ CD4⁺ (activated) and T1/ST2⁺ CD4⁺ (Th2) T cells in the lungs of HDM-exposed GATA eosinophil-deficient and WT mice relative to saline-exposed control mice (Figures 3E and 3F). Although the increases in activated T cells were comparable between HDM-exposed GATA and WT mice, we noted a slight reduction in the number of lung Th2 cells in the GATA group.

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

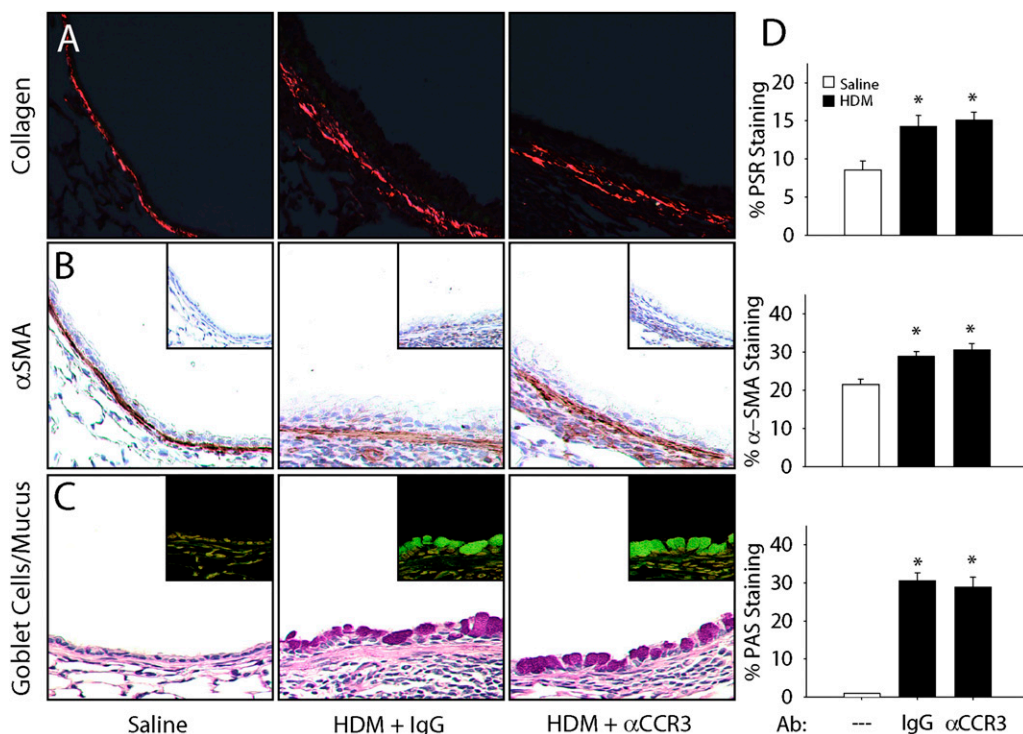


Figure 2. Impact of anti-CCR3 (α CCR3) treatment on house dust mite (HDM)-induced remodeling. Separate groups of mice were exposed to HDM (black bars) or saline (white bars) for 4 weeks. HDM-exposed mice were concurrently treated on Days 3, 6, 9, 13, 16, 19, 22, and 25 with α CCR3 or control IgG (0.5 mg per dose) antibody (Ab). Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM or saline exposure. (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition. (B) Immunohistochemistry for α -smooth muscle actin (α -SMA) indicating contractile elements in the airway wall (brown; insets show nonspecific staining in the corresponding negative control section). (C) Periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet

cells (magenta; insets show color-inverted image used for morphometric analysis). (D) Morphometric analysis of lung histology. Data represent the percentage of the area of interest that is stained with PSR, α -SMA, or PAS. All pictures were taken at 20 $\times$ magnification except insets in (C), which were at 40 $\times$ ($n = 5-7$ mice per group). Data are expressed as mean $\pm$ SEM and are from one of two independent experiments that yielded similar results. * $P < 0.05$ compared with the saline-exposed group. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test.

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

Impact of Eosinophil Deletion on HDM-induced Inflammation and Remodeling in *PHIL* Mice

In light of the reported differences pertaining to the role of eosinophils in the generation of OVA-induced allergic airway inflammation and hyperresponsiveness in Δ dblGATA versus *PHIL* eosinophil-deficient mice (11, 17, 21), we explored the effects of eosinophil deletion on HDM-induced responses in the *PHIL* strain of mice. We exposed *PHIL* eosinophil-deficient mice and WT littermate control mice to HDM for 5 weeks. Similar to GATA mice, *PHIL* mice developed significant airway inflammation in the BAL in response to 5 weeks of HDM exposure compared with saline-exposed *PHIL* control mice, although this response was markedly lower ($\sim 57\%$ fewer cells) than that observed in WT HDM-exposed littermate control mice (Figure 7A). Unlike what we observed in the GATA mice, the decrease in BAL inflammation in the *PHIL* mice was due not only to the absence of eosinophils but also to considerable reductions in the numbers of mononuclear cells and neutrophils ($\sim 43\%$ and $\sim 40\%$ fewer, respectively, relative to HDM-exposed WT mice) (Figures 7B–7D). Although eosinophils were virtually absent from the lung in-

flammatory infiltrate, we did not detect any decreases in the number of lung Th2 cells in *PHIL* HDM-exposed mice (Figures 7E and 7F). Equivalent increases in the levels of serum HDM-

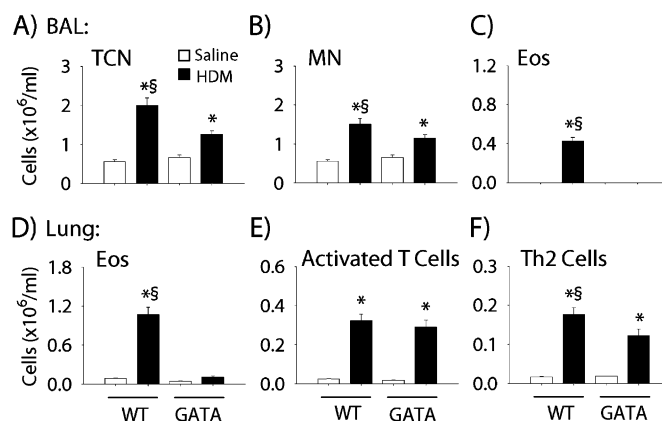


Figure 3. Impact of eosinophil deletion on house dust mite (HDM)-induced allergic airway inflammation in Δ dblGATA mice. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM (black bars) or saline (white bars) for 5 weeks and killed 72 hours after the last HDM exposure. (A–C) Total and differential cell count analysis showing total cell number (TCN), mononuclear cells (MN), and eosinophils (Eos) in the bronchoalveolar lavage (BAL). (D–F) Flow cytometric analysis of whole lung digests showing the number of Eos (CD45⁺ Gr-1^{low/int} SSC^{int/high}), activated T cells (CD45⁺, CD3⁺, CD4⁺, CD69⁺), and Th2 cells (CD45⁺, CD3⁺, CD4⁺, T1/ST2⁺). Gating strategies are described in Figure E1 in the online supplement. $n = 4$ to 8 mice per group. Data are expressed as mean $\pm$ SEM and are from one of two independent experiments that yielded similar results. * $P < 0.05$ compared with the *corresponding saline-exposed group and Δ HDM-exposed Δ dblGATA group. Statistical analysis was performed using two-way ANOVA with Tukey's *post hoc* test.

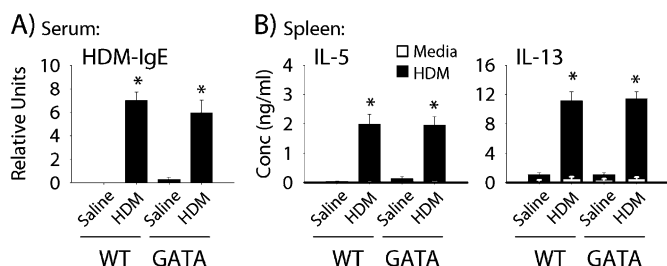


Figure 4. Impact of eosinophils on house dust mite (HDM)-specific Th2-associated adaptive immune responses. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM or saline for 5 weeks and killed 72 hours after the last exposure. (A) HDM-specific Th2-associated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

specific IgE were also observed between HDM-exposed *PHIL* and WT groups (Figure E3).

We investigated the consequence of eosinophil deletion in *PHIL* mice on the generation of HDM-induced remodeling. Airway remodeling was evident in HDM-exposed *PHIL* eosinophil-deficient mice after 5 weeks of HDM exposure (Figure 8 and Figure E4). However, modest decreases in peribronchial collagen deposition and smooth muscle thickness were observed relative to HDM-exposed WT mice, although, in the case of smooth muscle, significance was only achieved when the data were expressed as fold increase over baseline (Figures 8A and 8B).

No differences in goblet cells/mucus production were noted between *PHIL* and WT mice after 5 weeks of HDM (Figure 8C).

Impact of Eosinophil Deletion on HDM-induced Airway Hyperresponsiveness in GATA and *PHIL* Mice

We examined the effect of eosinophil deletion on lung function by evaluating airway (R_n) and tissue (G) resistance to increasing doses of methacholine (MCh) in GATA and *PHIL* mice. HDM-exposed WT and GATA mice displayed increases in airway and tissue resistance compared with corresponding saline-exposed control mice (Figures 9A and 9B). However, the extent of the increases in R_n over the corresponding saline-exposed control mice, appeared to be smaller in the GATA HDM-exposed group for most doses of MCh (Figure 9A). R_n was increased at only one dose of MCh in *PHIL* HDM-exposed mice relative to *PHIL* saline-exposed control mice, whereas R_n was increased at nearly every dose in the WT HDM-exposed group compared with WT saline control mice (Figure 9C). Increases in G were similar between WT and *PHIL* HDM-exposed groups (Figure 9D).

DISCUSSION

Numerous experimental studies have investigated the involvement of eosinophils in the remodeling response. The most common approach used to abolish eosinophilia has involved interference with IL-5 function. Of at least six studies using an IL-5-based approach, some have shown that, in the absence of eosinophils, OVA-induced remodeling is almost entirely abrogated (5, 10), whereas others observed only partial decreases (6, 8) or no effect of eosinophil ablation on remodeling (7, 9). The reasons for these discrepancies are not immediately apparent, but differences in the strain of mouse, protocols used, or

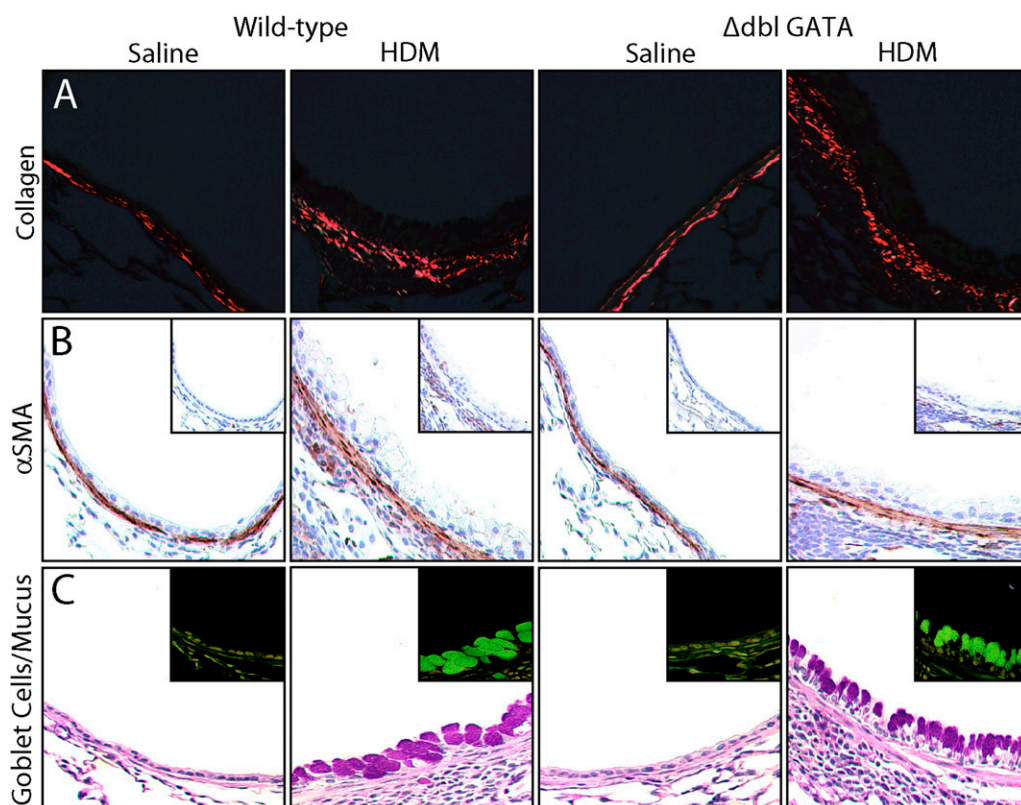


Figure 5. Histological evaluation of house dust mite (HDM)-induced allergic remodeling in Δ dblGATA mice. Separate groups of wild-type and Δ dblGATA eosinophil-deficient mice were exposed to HDM or saline for 5 weeks. Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition. (B) Immunohistochemistry for α -smooth muscle actin (α -SMA) indicating contractile elements in the airway wall (brown; insets show nonspecific staining in the corresponding negative control section). (C) Periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells (magenta; insets show color inverted image used for morphometric analysis). All pictures were taken at 20 $\times$ magnification except insets in (C), which were at 40 $\times$.

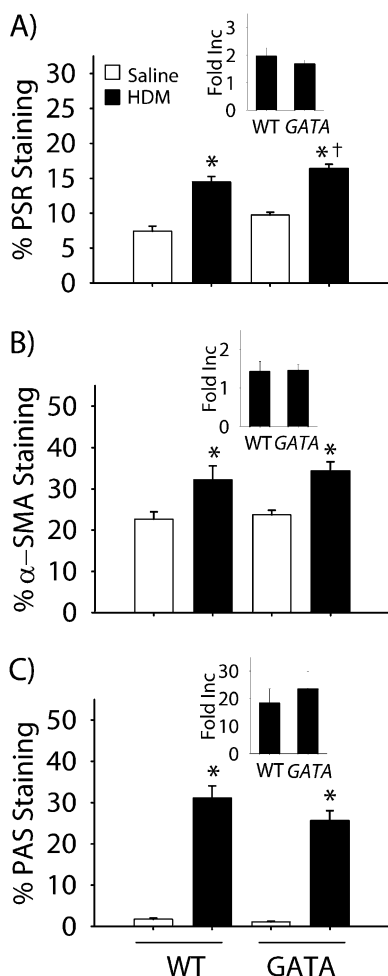


Figure 6. Morphometric analysis of the impact of eosinophil deletion on house dust mite (HDM)-induced remodeling in Δ dblGATA mice. Separate groups of wild-type (WT) and Δ dblGATA eosinophil-deficient mice were exposed to HDM (black bars) or saline (white bars) for 5 weeks. Lung tissues were obtained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

with the *corresponding saline-exposed group and †HDM-exposed WT group. Statistical analysis was performed using two-way ANOVA with Tukey's *post hoc* test (or *t* test for inset graphs).

approaches taken to eliminate eosinophils have been proposed as plausible explanations. Interestingly, GATA eosinophil-deficient mice subjected to chronic OVA exposure showed considerably reduced levels of total lung collagen and decreased numbers of total and proliferating smooth muscle cells, demonstrating that eosinophils significantly contributed to remodeling in that experimental system. Collectively, these findings have fueled, in spite of the notable discrepancies, the notion that eosinophils play a significant role in allergic airway remodeling.

Virtually all of the experimental findings discussed above have been conducted in the context of OVA-based models of allergic airway disease. Although these models have been instrumental to our understanding of disease pathogenesis, it is becoming increasingly evident that at least some of the underlying mechanisms that govern allergic airway inflammation and remodeling in those models differ from those induced by exposure to a CEA. For example, although TGF- β has been shown to be a critical mediator of OVA-induced remodeling (12–14), we have previously demonstrated that TGF- β is not required for the generation of remodeling induced by respiratory mucosal exposure to HDM (15), one of the most pervasive aeroallergens worldwide. Therefore, in the current study we explored the role of eosinophils in remodeling and adaptive immunity in a model induced by chronic HDM exposure. Using the α CCR3 Ab, we depleted approximately 80 to 85% of BAL

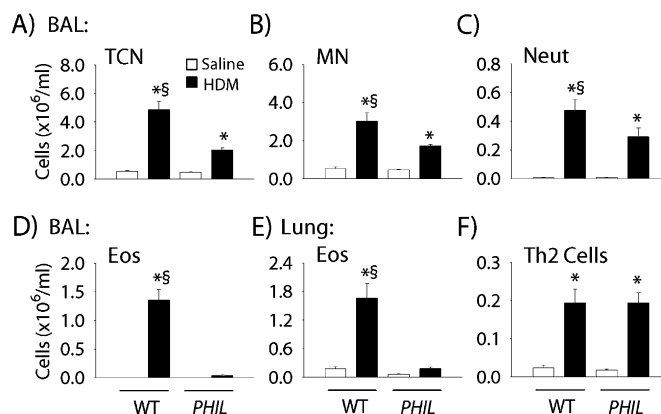


Figure 7. Impact of eosinophil deletion on house dust mite (HDM)-induced allergic airway inflammation in *PHIL* mice. Separate groups of wild-type (WT) littermate control and *PHIL* eosinophil-deficient mice were exposed to HDM (black bars) or saline (white bars) for 5 weeks and killed 72 hours after the last exposure. (A–D) Total and differential cell count analysis showing total cell number (TCN), mononuclear cells (MN), neutrophils (Neut), and eosinophils (Eos) in the bronchoalveolar lavage (BAL). $n = 3$ to 6 mice per group. (E and F) Flow cytometric analysis of whole lung digests showing the number of Eos (CD45⁺ Gr-1^{low/int} SSC^{int/high}) and Th2 cells (CD45⁺ CD3⁺ CD4⁺ T1/ST2⁺); gating strategies are described in Figure E1 in the online supplement. $n = 3$ to 5 mice per group. Data are expressed as mean $\pm$ SEM. $P < 0.05$ compared with the *corresponding saline-exposed group and §HDM-exposed *PHIL* group. Statistical analysis was performed using two-way ANOVA with Tukey's *post hoc* test.

and lung eosinophils over the course of a 4-week HDM exposure protocol. Although we did not achieve 100% depletion, eosinophils were reduced considerably, especially when the duration and intensity of the inflammatory response elicited by continuous HDM exposure is taken into account. This decrease in eosinophils had no impact on the development of HDM-induced remodeling. We also observed that remodeling developed, undiminished, in Δ dblGATA eosinophil-deficient mice after 5 weeks of HDM exposure. Additionally, goblet cell hyperplasia and mucus production were unaffected in HDM-exposed *PHIL* eosinophil-deficient mice. Moreover, although we documented significant decreases in collagen deposition and smooth muscle thickness in these mice compared with WT HDM-exposed littermates, collagen and smooth muscle were increased in the *PHIL* HDM-exposed group relative to saline-exposed control mice. In *PHIL* mice, the decreases in remodeling were associated not only with the absence of eosinophils but also with considerable reductions in the numbers of airway mononuclear cells and neutrophils, making it difficult to ascribe a direct role for eosinophils in the decreased remodeling response. Together, these data demonstrate that eosinophils are largely, if not entirely, dispensable for the development of HDM-induced remodeling under these experimental conditions.

The differential involvement of eosinophils on remodeling in OVA- versus HDM-based models provides further evidence of mechanistic differences underlying the development of remodeling and, likely, other processes between these two systems. There are a number of reasons that could account for this, although the two most evident, and perhaps the most essential, are the methods by which sensitization is achieved and the nature of the eliciting agents. Indeed, the incipient targets of mucosally delivered HDM are, in all likelihood, different from those reached by OVA administered intraperitoneally along

with an adjuvant. Moreover, HDM extracts, unlike OVA, contain a vast array of protein and nonprotein components, of which many are intrinsically able to trigger responses, immune and otherwise, that favor the generation of allergic sensitization (22–29). We surmise that this ability, in conjunction with the site of sensitization, enables HDM with the capacity to elicit a complex network of molecular signatures and mucosal immune responses fundamentally distinct from those elicited by OVA-based systems. Indeed, whereas uric acid/NALP3 inflammasome/IL-1 β -mediated mechanisms are thought to drive responses induced in conventional OVA-based systems (30, 31), MD-2/TLR-4/GM-CSF/TLSP-dependent mechanisms have been shown to be critical to the induction of HDM-induced responses (24, 28, 32). By extension, the inherent diversity among aeroallergens (e.g., roaches, fungi, and pollens) suggests that dissimilar responses could be expected from these as well. An important implication that arises from this premise is that the responses elicited in OVA-based systems may be, at least in part, misleading, not only from the perspective of our understanding of disease pathogenesis but also regarding our expectations of the therapeutic strategies that have been evaluated in them.

The contribution of eosinophils to allergic airway remodeling in humans is being investigated in clinical trials. The effect of short-term eosinophil depletion on extracellular matrix deposition was examined in a small group of patients with mild atopic asthma (33). The authors reported that anti-IL-5 treatment, which partially depleted pulmonary eosinophilia, was associated with significant decreases in some, but not all, of the remodeling-associated parameters examined (relative to pretreatment baselines). Although these findings suggest that eosinophil depletion had a beneficial impact on remodeling, there were a number of additional factors and study limitations noted by the authors that should be considered when interpreting these findings. In another study, normalization of sputum eosinophils, using a treatment guided by sputum cell counts and involving inhaled corticosteroids, was shown to have no effect on subepithelial collagen deposition in patients with mild to moderate asthma (34). Therefore, further investigation is required before the effects of eosinophil depletion on remodeling in humans can be clarified.

We also investigated the contribution of eosinophils to chronic HDM-induced inflammation and immunity. The substantial reduction in the number of BAL and lung eosinophils in HDM-exposed, α CCR3-treated mice had no impact on pulmonary inflammation; nor did it affect markers of HDM-specific Th2-sensitization (data not shown). Similarly, GATA eosinophil-deficient mice mounted a robust Th2-polarized immune inflammatory response after 5 weeks of HDM exposure. However, in addition to the absence of eosinophils, small decreases in BAL mononuclear and lung Th2 cells were noted in these mice. These findings indicate that eosinophils are involved in HDM-induced inflammation, although their contribution appears to be auxiliary rather than critical in nature. Furthermore, when considered in combination with our observations in the α CCR3 studies, the data suggest that even a small degree of eosinophilia is sufficient to mediate this enhancement. Although *PHIL* eosinophil-deficient mice displayed marked increases in BAL and lung inflammation (relative to saline-exposed control mice) after HDM exposure, we documented considerable reductions in BAL mononuclear cells and neutrophils, indicating that in these mice the absence of eosinophils was associated with a substantial decrease in the magnitude of the airway inflammatory response. That HDM-exposed *PHIL* mice had similar levels of HDM-specific IgE and numbers of lung Th2 cells as WT control mice indicates that this may have been due to

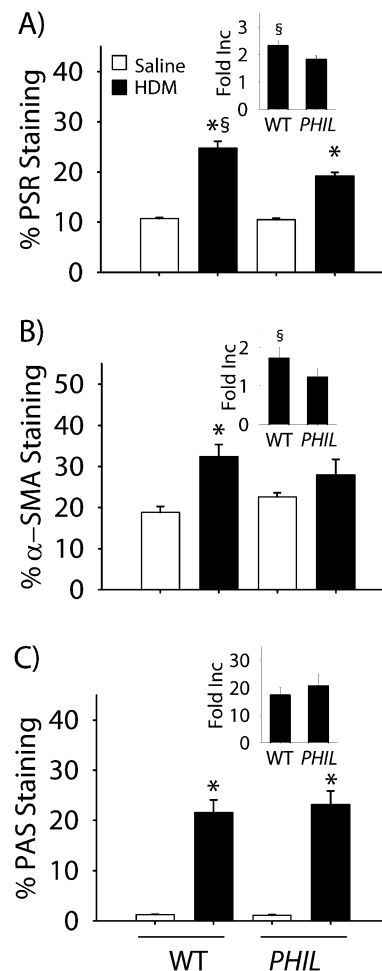


Figure 8. Morphometric analysis of the impact of eosinophil deletion on house dust mite (HDM)-induced remodeling in *PHIL* mice. Separate groups of wild-type (WT) littermate control and *PHIL* eosinophil-deficient mice were exposed to HDM (black bars) or saline (white bars) for 5 weeks. Lung tissues were obtained 72 hours after the last HDM exposure, and cross-sections were cut and stained with Picro Sirius red (PSR), α -smooth muscle actin (α -SMA), or periodic acid-Schiff (PAS). Morphometric analysis of lung histology was performed. Data represent the percentage of the area of interest that is stained with (A) PSR, indicating subepithelial collagen deposition; (B) α -SMA, indicating smooth muscle thickness; and (C) PAS, indicating mucus production by epithelial goblet cells. Inset graphs express data as the fold increase over the corresponding saline exposed group. $n = 3$ to 6 mice per group. Data are expressed as mean $\pm$ SEM. $P < 0.05$ compared with the *corresponding saline-exposed group and [§]HDM-exposed *PHIL* group. Statistical analysis was performed using two-way ANOVA with Tukey's *post hoc* test (or *t* test for inset graphs).

decreased cellular recruitment rather than impaired induction of adaptive immunity. It is unclear why decreases of such magnitudes were observed in *PHIL* but not in α CCR3-treated or GATA HDM-exposed mice, but the similarity of the findings among the latter two groups, along with the fact that all of these experiments were done in the same background strain of mice (BALB/c) and used the same exposure protocol, suggests that this observation may be, in some part, an idiosyncratic feature of the *PHIL* manipulation. Nonetheless, our data clearly demonstrate that eosinophils play little to no role in the establishment of HDM-induced allergic sensitization and that eosinophils play a modest, augmentative role in the generation of HDM-induced airway inflammation.

Our findings are in agreement with those documented by Humbles and colleagues, who observed undiminished OVA-specific Th2 sensitization, airway inflammation, and lung Th2 cells during the chronic and acute phases of their protocol in GATA eosinophil-deficient mice compared with WT control mice (11). In contrast, Jacobsen and colleagues reported that *PHIL* eosinophil-deficient mice subjected to an acute OVA-exposure model displayed dramatic reductions in BAL and lung lymphocytes (21) and markedly diminished splenocyte IL-13 production after OVA stimulation *in vitro* (21). The discrepancy between the Humbles and Jacobsen reports, which

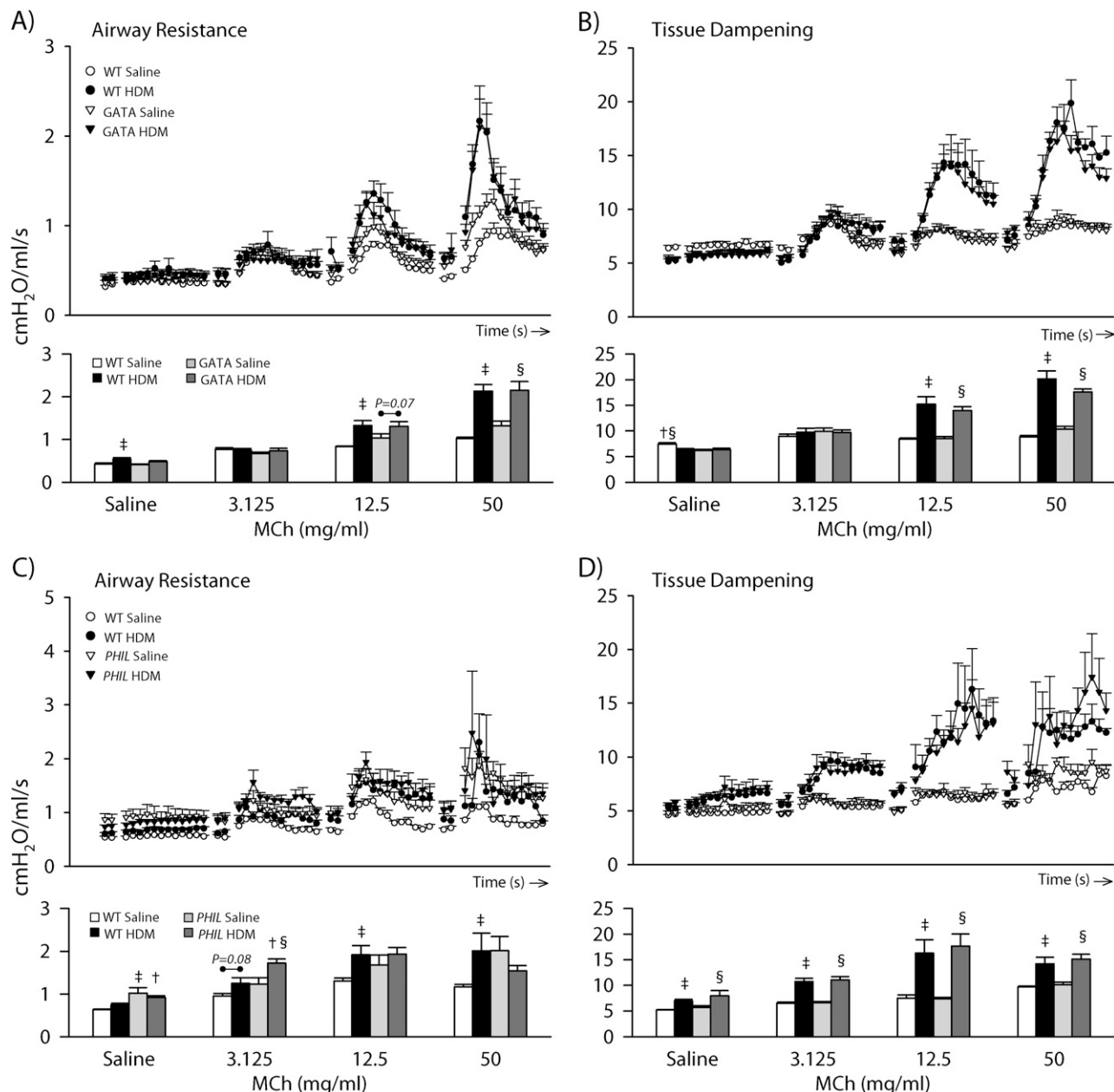


Figure 9. Impact of eosinophil deletion on house dust mite (HDM)-induced airway responsiveness to methacholine (MCh) in Δ dblGATA and *PHIL* eosinophil-deficient mice. Separate groups of wild-type (WT; circles) and eosinophil-deficient (triangles) mice were exposed to HDM (black symbols/black and dark gray bars) or saline (white symbols/white and light gray bars) for 5 weeks. (A and C) Airway resistance and (B and D) tissue damping were measured at increasing doses of MCh. Curves show the complete dose response data of a time-course involving two baseline measurements before nebulization of increasing doses of MCh, followed by 12 consecutive measurements after each dose. Bar graphs show the weighted average of peak respiratory resistance measured after saline and each of the MCh challenges. (A and B) $n = 6$ to 7 mice for saline-exposed and 8 to 9 mice for HDM-exposed groups. (C and D) $n = 5$ to 7 mice for saline-exposed and 7 to 10 mice for HDM-exposed groups. Data are expressed as mean $\pm$ SEM and are from one of three (for Δ dblGATA mice) and one of two (for *PHIL* mice) independent experiments that yielded similar results. $P < 0.05$ compared with the † WT saline-exposed group, ‡ WT saline-exposed group, and § WT HDM-exposed group. Statistical analysis was performed using two-way ANOVA with Tukey's *post hoc* test.

used similar OVA-based protocols, has recently been attributed, in large part, to differences in background strain (35). Walsh and colleagues observed that WT and BALB/c Δ dblGATA OVA-exposed mice developed similarly robust allergic inflammation, whereas C57BL/6 Δ dblGATA OVA-exposed mice displayed a marked reduction in this response. However, C57BL/6 Δ dblGATA OVA-exposed mice had similar serum levels of OVA-specific IgE as C57BL/6 WT mice, and splenic T cells from these mice responded equally well to

OVA restimulation *in vitro* as T cells from WT control mice, suggesting that the reduction in lung inflammation, in the absence of eosinophils, could be due to defects in cellular recruitment and not impaired sensitization. Thus, these findings do not explain the observation that *PHIL* mice show diminished splenocyte recall responses on account of their C57BL/6 background or the absence of eosinophils. The findings of Walsh and colleagues further imply that at least some component of the decreased immune responsiveness observed in

C57BL/6 *PHIL* mice may be a distinctive attribute of this specific mouse line. At variance with the notion that eosinophils are important mediators of allergic inflammation in C57BL/6 mice at all is an earlier report by Justice and colleagues showing that eosinophil ablation during only the challenge phase of their exposure protocol (via α CCR3 Ab treatment) had no impact on the influx of other leukocytes, including lymphocytes, to the airways (19).

Fulkerson and colleagues reported, in an acute model of *Aspergillus fumigatus*-induced allergic airway disease, that, in contrast to the findings of Humbles and colleagues in the acute phase of an OVA-based system, the number of airway lymphocytes was greatly reduced ($\sim 50\%$) in *Aspergillus*-exposed Δ dblGATA BALB/c mice (36). This finding, in conjunction with those described above, suggests that the nature of the allergen may also play a prominent role in determining the extent to which eosinophils contribute to allergic inflammation. We note that sensitization (i.e., Ag-specific Ig levels) was unaffected in *aspergillus*-exposed, eosinophil-deficient mice. Nor was it affected in another study where eosinophils were eliminated by interfering with IL-5, even when airway and lung mononuclear cells were decreased (37). Therefore, although the requirement for eosinophils in the development of allergic inflammation may depend on the strain of mouse and the nature of the allergen used, it does not appear as though eosinophils are critically required for the generation of allergic sensitization under any conditions.

Physiological evaluation of lung function in GATA and *PHIL* mice revealed that eosinophils contributed to a varying degree (minor in GATA mice and considerable in *PHIL*s) to the increases in airway resistance induced by chronic HDM exposure. Whether this contribution was directly mediated could not be ascertained because the absence of eosinophils in HDM-exposed GATA and *PHIL* mice was accompanied by varying decreases in other inflammatory cells. In fact, the decrease in airway resistance appeared to track more closely with the decrease in mononuclear cells than with the absence of eosinophils. Historically, the literature pertaining to the impact of eosinophils on airway responsiveness in experimental models has been highly conflicted. Of the nearly two dozen studies that have been conducted, approximately half have reported that eosinophils play a role in this process, and half have reported that they do not (38). The functional consequence of eosinophils in human allergic asthma has been investigated in several clinical trials. Collectively, the results of these studies suggest that eosinophils play a marginal role in airway hyperresponsiveness and lung dysfunction in the overall population of patients with allergic asthma and that, in certain subsets of patients with asthma, IL-5 neutralization and, presumably, eosinophil blockade significantly reduces exacerbations (39–43).

A considerable body of experimental research, largely in OVA-based models, has instigated the notion that eosinophils are principal mediators of allergic inflammation and airway remodeling. In the present study, we have demonstrated that eosinophils are dispensable for the development of adaptive Th2 immunity and airway remodeling induced by the prolonged exposure to HDM. Rather than fostering a futile dichotomy (OVA vs. HDM), our findings highlight a simple and perhaps underappreciated concept: that the role of a given cell or molecule for that matter is contextual and, therefore, that caution must be applied to not exert unqualified universal forecasts. From this perspective, the divergence of findings regarding the role of eosinophils on airway inflammation, Th2 immunity, and airway remodeling in systems using OVA and CEAs, such as HDM or fungal antigens, may not be contradictory but rather may be an expression of the heterogeneity that underlies allergic re-

sponses. In this regard, it has now become clear that allergic airway diseases encompass an array of distinct phenotypes (44, 45) or “endotypes,” as referred to by Anderson (44). Thus, the diversity of responses observed in experimental systems may be a consonant, but fractional, fitting to the heterogeneity of human asthma. Yet, an unselective attachment to experimental simplicity and habitual practices may alienate experimental research from the complexity of human biological processes and foster deceiving interpretations with respect to disease pathogenesis and therapeutic expectations.

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Table E1

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Table E1. Impact of anti-CCR3 treatment on eosinophilia in the blood, BAL and lungs of HDM-exposed mice. Area under the curve was calculated for the curves shown in *Figures 1A, B and C* and used to assess the overall extent to which anti-CCR3 treatment decreased eosinophilia during weeks 2-4 of HDM exposure.

	Eosinophils - Area Under the Curve [*]			% Decrease (relative to Saline) [†]
	Saline	HDM+IgG	HDM+ α CCR3	
Blood	4.5	12.6	1.0	143.4
BAL [‡]	0.0	75.2	16.7	77.8
Lung	9.4	29.9	12.3	85.7

^{*}The calculation of the area under the curves were derived from mathematical equations generated from the mean experimental data using curve-fitting techniques from FindGraph software (UNIPHIZ Lab, Vancouver, Canada). The calculation of the areas under each curve were determined by encoding the equations in Mathematica 6.0 (Wolfram, Chicago, U.S.A).

[†]% Decrease was calculated as follows: $((AUC_{HDM+IgG} - AUC_{Saline}) - (AUC_{HDM+\alpha CCR3} - AUC_{Saline})) / (AUC_{HDM+IgG} - AUC_{Saline}) * 100$

[‡]Abbreviations: BAL - bronchoalveolar lavage; α CCR - anti-CCR3; AUC - area under the curve

Figure E1

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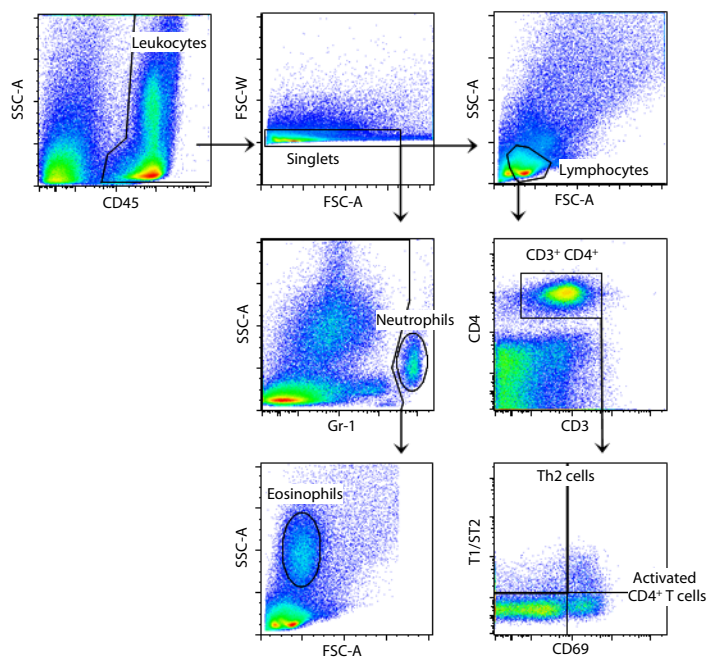


Figure E2

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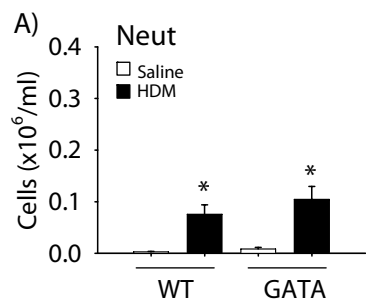


Figure E3

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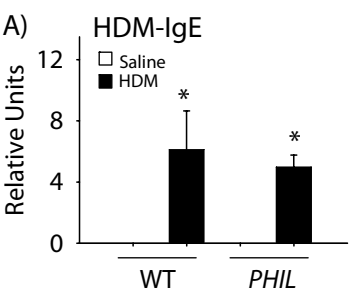


Figure E4

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