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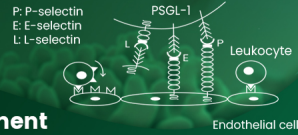
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A Causative Relationship Exists Between Eosinophils and the Development of Allergic Pulmonary Pathologies in the Mouse¹

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Asthma and mouse models of allergic respiratory inflammation are invariably associated with a pulmonary eosinophilia; however, this association has remained correlative. In this report, a causative relationship between eosinophils and allergen-provoked pathologies was established using eosinophil adoptive transfer. Eosinophils were transferred directly into the lungs of either naive or OVA-treated IL-5^{-/-} mice. This strategy resulted in a pulmonary eosinophilia equivalent to that observed in OVA-treated wild-type animals. A concomitant consequence of this eosinophil transfer was an increase in Th2 bronchoalveolar lavage cytokine levels and the restoration of intracellular epithelial mucus in OVA-treated IL-5^{-/-} mice equivalent to OVA-treated wild-type levels. Moreover, the transfer also resulted in the development of airway hyperresponsiveness. These pulmonary changes did not occur when eosinophils were transferred into naive IL-5^{-/-} mice, eliminating nonspecific consequences of the eosinophil transfer as a possible explanation. Significantly, administration of OVA-treated IL-5^{-/-} mice with GK1.5 (anti-CD4) Abs abolished the increases in mucus accumulation and airway hyperresponsiveness following adoptive transfer of eosinophils. Thus, CD4⁺ T cell-mediated inflammatory signals as well as signals derived from eosinophils are each necessary, yet alone insufficient, for the development of allergic pulmonary pathology. These data support an expanded view of T cell and eosinophil activities and suggest that eosinophil effector functions impinge directly on lung function. *The Journal of Immunology*, 2003, 170: 3296–3305.

Allergic asthma is a syndrome with marked heterogeneity characterized by chronic immune-mediated responses in the lung, leading to airway changes (e.g., mucus hypersecretion and airway epithelial pathology) and pulmonary dysfunction (e.g., reversible airflow limitations and airway hyperresponsiveness (AHR)²) (1, 2). Despite differences occurring in underlying inflammatory responses and the presentation of symptoms, the presence of eosinophils in the airway mucosa and lumen has been recognized even in the earliest studies (see, for example, Ref. 3) as a defining feature of this syndrome (70–90% of reported cases (4)). This pulmonary eosinophilia correlates with the histopathological changes occurring in asthma patients and is associated with the development of lung dysfunction even in mild cases (4, 5). The implicit conclusion of these correlative studies is that airway pathology is a concomitant consequence of allergen-induced pulmonary eosinophilia.

Mouse models of allergic respiratory inflammation have also contributed to the hypothesis that allergen-induced pulmonary eosinophilia is linked to the onset/progression of lung pathology. Knockout models deficient of IL-5 and “conditional knockouts” using anti-IL-5 Abs have been particularly informative. These studies have demonstrated that allergen-mediated pulmonary pathologies also correlate with an induced pulmonary eosinophilia, including airway mucus accumulation (6, 7) and AHR (8–10). However, defining a causative relationship between allergen-induced eosinophilia and pulmonary pathologies in the mouse has remained an elusive goal. Moreover, numerous studies have attempted to disassociate these phenomena. For example, studies have suggested that CD4⁺ T cells, and not eosinophils, are causatively linked to airway histopathology (e.g., airway mucus accumulation (11)). In addition, the lack of a relationship between eosinophils, airway mucus, and development of AHR was also shown in a study using an α_4 -integrin antagonist (12) and still yet other studies (see, for example, Refs. 13–17) have attempted to disassociate allergen-induced pulmonary eosinophilia from lung dysfunction (e.g., AHR). Thus, regardless of the preponderance of evidence, the relationship between eosinophils and allergen-mediated pulmonary pathologies has remained correlative with ongoing debates in both humans (see, for example, Ref. 18 vs Ref. 19) and mice (see, for example, Ref. 8 vs Ref. 16) as to the physiological relevance of this relationship.

Previous reports assessing immune-mediated inflammatory responses in the mouse have been greatly aided by the availability of neutralizing Abs or gene knockout animals deficient for a given inflammatory signal or cell type. Moreover, in combination with adoptive transfer, these studies have often permitted a determination of the unique role(s) of the signal/cell type by assessing pulmonary inflammation in its presence vs absence. Several experimental limitations have prevented the use of this strategy to assay

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² Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage, i.n., intranasal; IT, intratracheal; MBP, major basic protein; Penh, enhanced pause.

directly the significance of the induced pulmonary eosinophilia in allergen challenge models. These limitations include the lack of uniquely eosinophil-deficient mice and experimental strategies to isolate and adoptively transfer large numbers of eosinophils into syngeneic mice. The creation of IL-5^{-/-} mice, and/or the use of anti-IL-5 Abs, have provided model systems that are nearly devoid of eosinophils. Thus, the loss of allergen-mediated pathologies in IL-5-deficient animals appears to link eosinophils to the development of these pulmonary pathologies. However, strategies targeting IL-5 to ablate eosinophils have inherent ambiguities because of activities this cytokine has on other cell types, including B cells (20), T cells (7, 21–23), and, potentially, airway smooth muscle activity (24). That is to say, is the loss of allergen-induced pathology in IL-5-deficient mice a consequence of effects on eosinophils and/or effects on these other IL-5 responsive cells? Nonetheless, despite this limitation, the availability of an eosinophil adoptive transfer strategy in the mouse would permit the restitution of a pulmonary eosinophilia in IL-5^{-/-} mice and, in turn, possibly identify a causative link between the presence of pulmonary eosinophils and allergen-mediated pathologies. To this end, a strategy to isolate and adoptively transfer eosinophils was developed that uses an IL-5 transgenic mouse line (NJ.1638 (Ref. 21)) as the source of eosinophils and syngeneic IL-5^{-/-} mice as the adoptive transfer recipients. The data demonstrate that the transfer of eosinophils into IL-5^{-/-} mice restores pulmonary eosinophilia (i.e., in both the airway lumen and peribronchial areas) to wild-type levels and results in the recovery of allergic respiratory pathologies. Significantly, allergen-treated recipients depleted of CD4⁺ T cells failed to develop pulmonary pathologies following eosinophil adoptive transfer. Thus, these data reveal a synergy between CD4⁺ T cells and eosinophils, both of which are required, but alone are insufficient, for the development of the pathologies associated with allergic respiratory inflammation.

Materials and Methods

Mice

IL-5-deficient (IL-5^{-/-}) knockout animals on a C57BL/6J background were a kind gift of M. Kopf (then at the Basel Institute of Immunology, Basel, Switzerland) (25). Since receiving these animals the mice have been continuously backcrossed with C57BL/6J mice (>10 generations) purchased from The Jackson Laboratory (Bar Harbor, ME). Genotypes of mice derived from these crosses were determined by PCR of tail DNA, detecting the presence of the mutant IL-5 allele. Control C57BL/6J mice were obtained from The Jackson Laboratory, and all procedures were conducted on mice 8–12 wk of age maintained in ventilated microisolator cages housed in a specific pathogen-free animal facility. The sentinel mice within this animal colony were negative for the presence of known mouse pathogens. Protocols and studies involving animals were conducted in accordance with National Institutes of Health and Mayo Clinic Foundation guidelines.

Isolation of eosinophils

Peripheral blood eosinophils were isolated from IL-5 transgenic mice which constitutively express this cytokine exclusively from mature T cells using an enhancer/promoter construct derived from the mouse CD3 δ gene (transgenic line: NJ.1638 (Ref. 21)). NJ.1638 mice are maintained on a C57BL/6J genetic background by continuous backcross (>12 generations) with mice purchased from The Jackson Laboratory. The average circulating eosinophil counts in 4- to 7-mo-old animals are >100,000 eosinophils/mm³ of blood, representing 50% of total white blood cells. Pure populations of eosinophils for adoptive transfer were recovered as described earlier (26). Briefly, peripheral blood (tail bleeds or terminal bleeds from cardiac puncture) was collected into PBS containing 20 U/ml heparin, layered onto a single-step Percoll gradient, and centrifuged (45 min, 2000 \times g) at 4°C. The buffy coat, containing almost exclusively lymphocytes and eosinophils, was freed of contaminating red blood cells by hypotonic lysis and eosinophils were isolated by a negative selection strategy, removing B cells and T cells with Ab conjugated magnetic beads (MACS, Miltenyi Biotec, Auburn, CA) specific for CD45-R (B220) and CD90 (Thy 1.2),

respectively. The remaining cells were washed twice with RPMI 1640 and resuspended in PBS, maintaining these leukocytes at 4°C before use. The purity of the recovered eosinophils was determined by visual examination of DiffQuick stained cytopsin preparations and was typically >98.5% (monocytes (0.5%) and neutrophils (1%) comprise the contaminating cell populations). Eosinophil viability was determined by trypan blue exclusion and was always >99.6%. Assessments of chemotaxis and morphological characterizations of the recovered eosinophils revealed that the isolation was not accompanied by eosinophil activation (see, for example, Ref. 26). The eosinophils purified from NJ.1638 mice did not display levels of chemotaxis significantly different relative to eosinophils from unpurified white blood cell preparations, they generated very little to no reactive oxygen species, and displayed electron microscopic profiles consistent with intact resting cells (i.e., no evidence of degranulation or changes in granule morphology, including release of major basic protein (MBP), was observed).

OVA sensitization/aerosol challenge and intratracheal (IT) transfer of eosinophils

Mice were sensitized and challenged with chicken OVA as previously described (27). Briefly, mice were sensitized by an i.p. injection (100 μ l) of 20 μ g chicken OVA (Sigma-Aldrich, St. Louis, MO) emulsified in Imject alum (2.25 mg Al(OH)₃/2 mg Mg(OH)₂) (Pierce, Rockfield, IL) on days 0 and 14. Mice were subsequently challenged with an aerosol generated from 1% OVA in saline (OVA), or saline alone, for 20 min by ultrasonic nebulization (DeVilbiss, Somerset, PA) on days 24, 25, and 26. The mice were assessed for pulmonary cellular infiltrates, histopathologies, bronchoalveolar lavage (BAL) cytokine levels, and lung function on day 28.

The adoptive transfer of eosinophils and/or vehicle control (PBS) into the lungs of mice was accomplished in subject animals lightly anesthetized with Ketamine-HCL (40 μ g/gram body weight; Ketaset Fort Dodge Animal Health, Fort Dodge, IA) before IT instillation. Anesthetized animals were placed in a holder physically supporting the mice in an upright position. The trachea was cleared by holding the tongue to one side with a pair of forceps and a suspension of eosinophils (10⁷ eosinophils/20 μ l) was instilled (alternatively 10⁵ eosinophils/20 μ l were instilled in some experiments); control animals received 20 μ l of PBS, using a gel loading pipette-tip (Finepoint LTS (catalog no. RT-L10F) Rainin Instrument, Woburn, MA). A time line of these experimental manipulations is presented in Fig. 1, including both the OVA sensitization/challenge and adoptive transfer schedules. On the days that mice received both an OVA aerosol challenge and an IT instillation (i.e., days 24, 25, and 26) these procedures were staggered with the OVA aerosol challenge occurring in the morning and the instillation occurring the same day, generally 4–6 h later.

Cytokine assays

Cytokine levels in BAL fluid were determined by ELISA. Mouse IFN- γ , IL-4, IL-5, and IL-13 levels were assessed using immunoassay kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. The limits of detection for each cytokine assay were 5–10 pg/ml.

Ab-mediated neutralization of IL-5 or depletion of CD4⁺ T cells

Additional groups of OVA-treated IL-5^{-/-} mice were also administered either rat anti-mouse IL-5 (TRFK-5 (28)) or rat anti-mouse CD4 (GK1.5 (29)) before IT instillation of eosinophils.

Administration of anti-IL-5 mAbs. TRFK-5 was administered using an experimental protocol that abolishes both the pulmonary eosinophilia and the AHR occurring in OVA-treated wild-type mice. TRFK-5 was administered systemically (i.e., i.p. injection of 100 μ g) as well as locally (i.e., intranasal (i.n.) administration of 50 μ g in 30 μ l of saline) into lightly

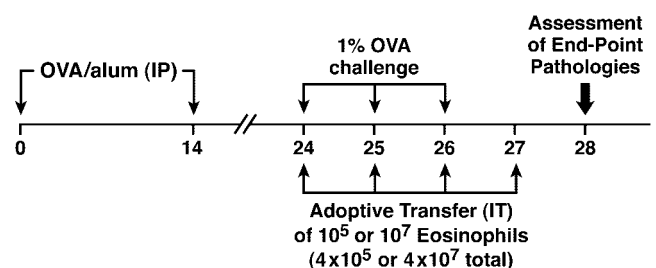


FIGURE 1. Time line representation of the OVA protocol used and the IT adoptive transfer of eosinophils.

anesthetized animals on day 23 of the OVA protocol described in Fig. 1. In addition, 2 h before each OVA airway challenge (and ~6–8 h before IT instillation of eosinophils) TRFK-5 was administered i.n. on days 24, 25, and 26. On day 27, ~6–8 h before IT instillation of eosinophils, a final i.n. dose of TRFK-5 was administered. Control groups of animals received nonspecific rat IgG (Sigma-Aldrich).

Administration of anti-CD4 mAbs. CD4⁺ cells were depleted from OVA-treated IL-5^{-/-} mice using a protocol first described by Gavett et al. (30). GK1.5 was administered via the peritoneal cavity (0.5 mg/100 μ l), control groups received nonspecific rat IgG, three days before the first OVA aerosol challenge (day 21, Fig. 1).

Assessments of allergic airway inflammation: pulmonary cellular infiltrates and Ag-induced histopathologies

The cellularity of the airspaces was determined by BAL as previously described (31). Immunohistochemical detection of eosinophils in lung tissue sections (4 μ m) was performed using a rabbit polyclonal antisera specific for mouse MBP (32). Staining was performed with diaminobenzidine-peroxidase detection reagents as described in earlier studies (23, 27, 31).

Histopathologic changes of the airways, including mucus cell content of the epithelium, were assessed in paraffin-embedded lungs following formalin fixation (lungs were inflated with a fixed volume (0.5 ml) of fixative). Parasagittal sections (4 μ m) were stained with periodic acid-Schiff's reagent and counterstained with hematoxylin/methyl green to assess mucus production and goblet cell metaplasia. The mucus content of airways (both proximal and distal) were assessed by brightfield microscopy ($n = 5$ mice per group) using an image analysis software program (Image ProPlus, Silver Spring, MD) to derive an airway mucus index that is reflective of both the amount of mucus per airway and the number of airways affected (7).

Determination of AHR in response to methacholine challenge

AHR was assessed by whole-body plethysmography, inducing airflow obstruction with a methacholine aerosol (32, 33). This methodology (Buxco Electronics, Troy, NY) relies on pressure differences between a chamber containing conscious/unrestrained mice and a reference chamber to extrapolate minute volume, tidal volume, breathing frequency, and enhanced pause (Penh). Penh is a dimensionless parameter that is a function of total pulmonary airflow in mice (i.e., the sum of the airflows in the upper and lower respiratory tracts) during the respiratory cycle of the animal and closely correlates with airway resistance as measured by traditional invasive techniques using ventilated animals (33). Dose-response data were plotted either as raw Penh values or percent baseline Penh vs the log₁₀ of

the methacholine solution (milligrams per milliliter) used to generate the aerosol.

Statistical analysis

Data presented are the means (\pm SE). Statistical analysis was performed on parametric data using Student's *t* tests with differences between means considered significant when $p < 0.05$.

Results

Adoptive transfer of eosinophils into the lungs of IL-5^{-/-} mice reconstitutes a pulmonary eosinophilia equivalent to wild-type mice

Previous studies have demonstrated that the IL-5 deficiency associated with IL-5^{-/-} mice led to a significant reduction of pulmonary eosinophils (relative to wild-type mice) following allergen sensitization/challenge, particularly the eosinophilia of the airway lumen (6, 8–10). A strategy to restore this pulmonary eosinophilia was developed to assess the relative contribution of lung eosinophils to pathologic changes occurring in OVA treated mice. An IL-5 transgenic mouse line constitutively expressing IL-5 from circulating T cells (line NJ.1638 (Ref. 21)) was used to isolate pure (>98%) populations of mouse peripheral blood eosinophils with nearly 100% viability. The adoptive transfer protocol used is diagrammed in Fig. 1 and includes the IT instillation of 1×10^7 eosinophils on each of the three aerosol challenge days of the OVA protocol as well as the day following the last challenge (i.e., adoptive transfer of a total of 4×10^7 eosinophils). The transfer of this number of eosinophils was based on the $1\text{--}3 \times 10^7$ eosinophils present in the lungs of OVA-treated wild-type mice as demonstrated in collagenase-digested lung preparations (Ref. 34 and our unpublished data). The BAL data in Fig. 2 show that the adoptive transfer of eosinophils directly into the airways of OVA-treated IL-5^{-/-} mice increases airway lumen eosinophils to levels equivalent of OVA-treated wild-type mice. The induced BAL eosinophilia following transfer was not dependent on OVA sensitization/aerosol challenge of the recipient mice, although fewer BAL eosinophils were consistently observed in naive vs OVA-treated

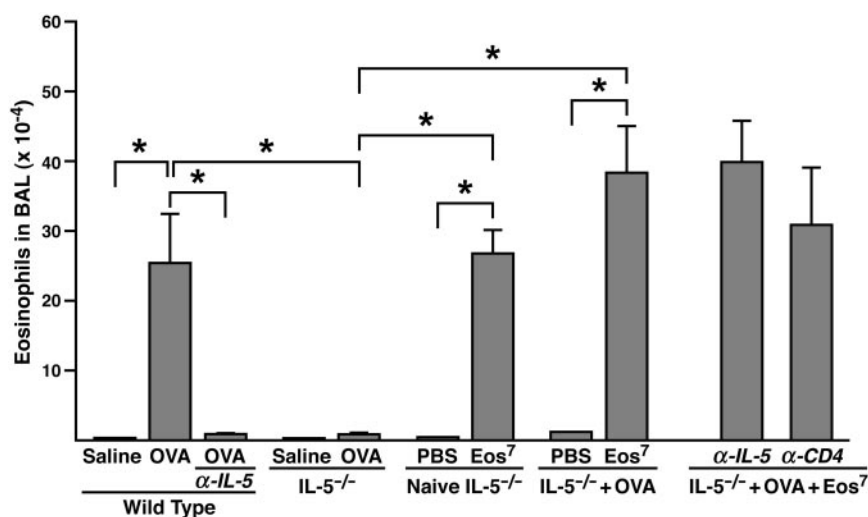


FIGURE 2. Adoptive transfer of eosinophils into the lungs of IL-5^{-/-} mice restores an airway eosinophilia equivalent to OVA-treated wild-type animals. The eosinophil levels in BAL fluid from wild-type and IL-5^{-/-} mice following either a saline or OVA aerosol challenge were compared with naive vs OVA aerosol challenged IL-5^{-/-} mice following IT adoptive transfer of 4×10^7 eosinophils (Eos⁷) or PBS vehicle control. Additional control groups shown include OVA-treated wild-type mice administered anti-IL-5 mAb TRFK-5 (OVA/ α -IL-5), OVA-treated IL-5^{-/-} mice concurrently administered TRFK-5 before IT instillation of eosinophils (α -IL-5/IL-5^{-/-} + OVA + Eos⁷), and OVA-treated IL-5^{-/-} mice concurrently administered GK1.5 to deplete CD4⁺ T cells before IT instillation of eosinophils (α -CD4/IL-5^{-/-} + OVA + Eos⁷). The eosinophils found in the BAL of each animal cohort are expressed as the product of the total number of cells recovered and the percentages of eosinophils derived from differentials (Wright's stained cytocentrifuge preparations) of 300 cells. Data represent mean values \pm SE ($n = 5\text{--}10$ animals/group). *, $p < 0.05$.

IL-5^{-/-} animals. In addition, the resultant BAL eosinophilia did not occur as a consequence of the adoptively transferred wild-type eosinophils reconstituting IL-5 in the knockout mice. Coadministration of anti-mouse IL-5 mAb (TRFK-5) had no effect on the BAL eosinophilia of OVA-treated IL-5^{-/-} mice. Moreover, the depletion of CD4⁺ cells in OVA-treated IL-5^{-/-} mice also failed to affect the BAL eosinophilia induced following adoptive transfer.

The distribution of tissue eosinophils in the lungs of mice following adoptive transfer was determined by immunohistochemistry using a rabbit anti-mouse MBP polyclonal antiserum (32). In addition to confirming the significant loss of tissue eosinophils in OVA-treated IL-5-deficient mice (i.e., either Ab depleted wild-type or IL-5 knockout) relative to OVA-treated wild-type animals, these data demonstrate that IT instillation of eosinophils into OVA-treated IL-5^{-/-} mice reconstitutes the peribronchial eosinophilia occurring in OVA-treated wild-type animals (Fig. 3). The distribution of tissue eosinophils following adoptive transfer was

equivalent to the pattern observed in OVA-treated wild-type mice with the exception of a smaller, yet significant, perivascular eosinophilia. The identity of these tissue eosinophils as originating from the adoptive transfer was demonstrated by ex vivo labeling the eosinophils with a fluorescent tag before IT instillation (data not shown). Quantitative assessments of peribronchial eosinophils surrounding individual airways in each group of mice are shown in Fig. 4. Tissue eosinophil accumulation was not dependent on OVA sensitization/aerosol challenge although tissue eosinophil numbers in naive mice were consistently lower than OVA-treated IL-5^{-/-}. These data show that the adoptive transfer of eosinophils into OVA-treated IL-5^{-/-} mice reconstituted eosinophil levels to those observed in wild-type animals. Moreover, this tissue eosinophilia did not occur as a consequence of reconstituting IL-5 in the knockout mice. In addition, the depletion of CD4⁺ cells in OVA-treated IL-5^{-/-} mice resulted in a nominal, yet significant, decrease in the observed tissue eosinophilia occurring after adoptive transfer.

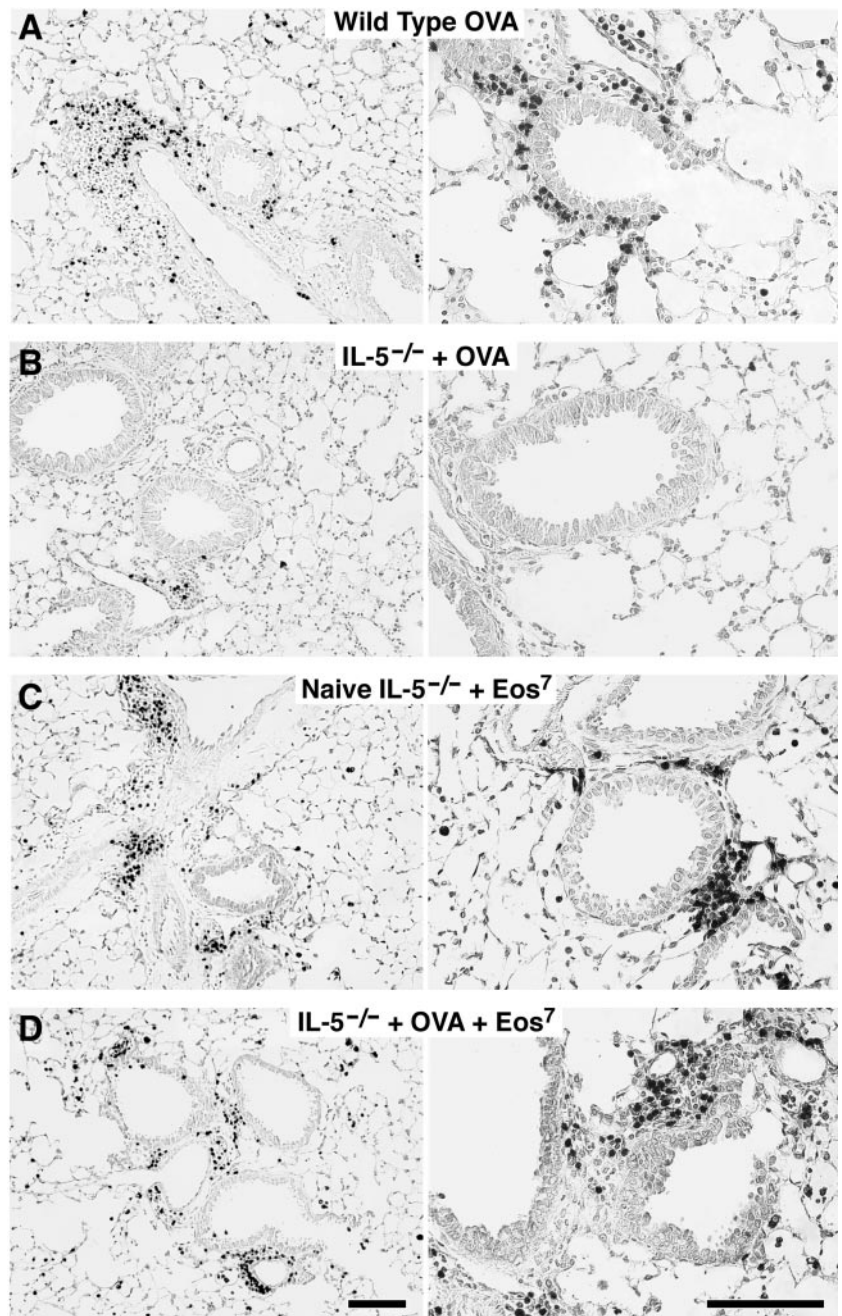
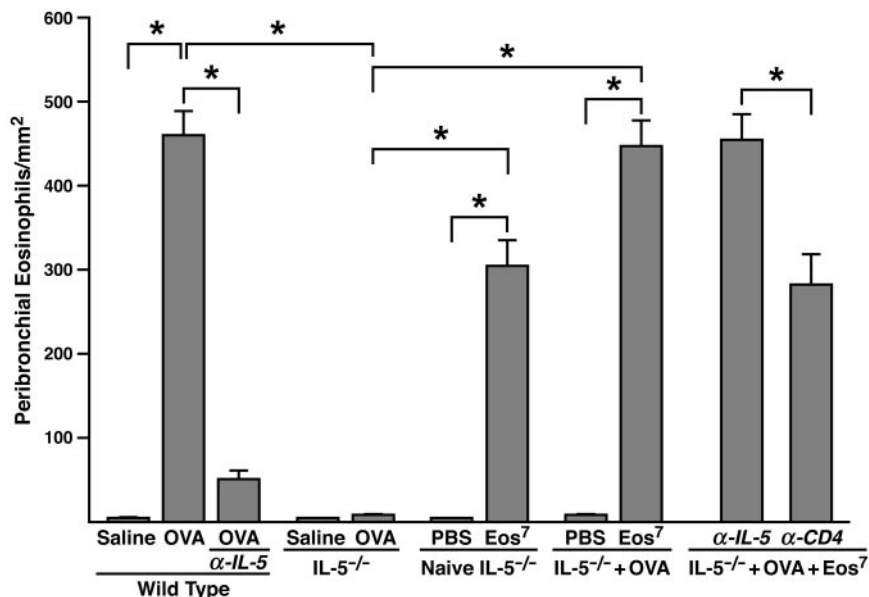


FIGURE 3. Adoptive transfer of eosinophils into the lungs of IL-5^{-/-} mice results in a pulmonary tissue eosinophilia equivalent to the spatial distribution of eosinophils in OVA-treated wild-type animals. The accumulation of eosinophils to the lungs of wild-type and IL-5^{-/-} mice was assessed by immunohistochemistry using a rabbit polyclonal antiserum specific for mouse MBP (32). The photographs on the *left* of each panel are low magnification views of the lung parenchyma. The photographs on the *right* of each panel are representative examples at high magnification of the eosinophil accumulation typically found in peribronchial areas. *A*, OVA sensitized/challenged mice (wild-type OVA); *B*, OVA sensitized/challenged IL-5 knockout mice (IL-5^{-/-} + OVA); *C*, Naive IL-5^{-/-} mice following IT transfer of eosinophils (Naive IL-5^{-/-} + Eos⁷); *D*, OVA-treated IL-5^{-/-} mice following IT transfer of eosinophils (IL-5^{-/-} + OVA + Eos⁷). Bars = 100 μ m.

FIGURE 4. The pulmonary tissue eosinophilia following adoptive transfer into IL-5^{-/-} mice is quantitatively equal to the eosinophilia occurring in OVA-treated wild-type animals. Pulmonary tissue eosinophils were assessed as a function of peribronchial area (square millimeters). The animal groups assessed include wild-type and IL-5^{-/-} mice following saline or OVA challenge vs naive and OVA-treated IL-5^{-/-} mice following IT instillation of 4×10^7 eosinophils (Eos⁷) or PBS vehicle control. Additional control groups shown include OVA-treated wild-type mice administered anti-IL-5 mAb TRFK-5 (OVA/ α -IL-5), OVA-treated IL-5^{-/-} mice concurrently administered TRFK-5 (IL-5^{-/-} + OVA + Eos⁷/ α -IL-5) before IT instillation of eosinophils, and OVA-treated IL-5^{-/-} mice concurrently administered GK1.5 to deplete CD4⁺ T cells before IT instillation of eosinophils (IL-5^{-/-} + OVA + Eos⁷/ α -CD4). The values presented are means (\pm SE) derived from two to five sections per mouse ($n = 5-7$ mice per group). *, $p < 0.05$.



OVA-induced airway epithelial mucus production is dependent, in part, on the presence of pulmonary eosinophils

An often overlooked consequence of manipulating *in vivo* IL-5 levels during allergen challenge is a significant change in airway mucus production (6, 7). These changes in airway epithelial mucus production are evident in either OVA-treated wild-type mice administered TRFK-5 or OVA-treated IL-5^{-/-} mice. In both cases, airway mucus accumulation decreased ~50% relative to OVA-treated wild-type animals (Figs. 5 and 6). IT instillation of eosinophils into OVA-treated IL-5^{-/-} mice (restoring pulmonary eosinophil numbers to wild-type levels) eliminates this decrease in airway epithelial mucus such that no differences are observed relative to OVA-treated wild-type mice (Figs. 5 and 6). This recovery of airway mucus was dependent on the restitution of pulmonary eosinophils to wild-type levels. The partial restitution occurring following transfer of 4×10^5 eosinophils, which nonetheless induced an airway and tissue eosinophilia ~10 times OVA-treated IL-5^{-/-} mice ($0.58 \pm 0.19 \times 10^4$ vs $0.07 \pm 0.03 \times 10^4$, respectively), was insufficient to recover the mucus production observed in OVA-treated wild-type mice (Fig. 5). The induction of airway epithelial mucus was not a nonspecific consequence of instilling eosinophils into the lungs of IL-5^{-/-} mice. The instillation of eosinophils into naive IL-5^{-/-} had no effect on mucus production despite the enormous BAL and peribronchial eosinophilia induced in these mice (Fig. 6). Interestingly, the increases in intracellular epithelial mucus following eosinophil adoptive transfer into OVA-treated IL-5^{-/-} mice were accompanied by increases in BAL Th2 cytokine levels (Fig. 7). These eosinophil-induced changes included the appearance of airway IL-5, a significant increase in BAL IL-4 levels (relative to OVA-treated IL-5^{-/-} mice), and an increase in BAL IL-13 levels. IFN- γ levels remained below detection limits in all the control/experimental groups examined. The eosinophil-associated induction of mucus, however, was not dependent on the elaboration of IL-5 levels in IL-5^{-/-} mice as concurrent administration of TRFK-5 to OVA-treated IL-5^{-/-} animals had no effects on the mucus index (Fig. 6). Significantly, the recovery of intracellular epithelial mucus accumulation following adoptive transfer of eosinophils did not occur in OVA-treated IL-5^{-/-} mice depleted of CD4⁺ T cells (Fig. 6). These data demonstrate that both eosinophils and OVA-induced inflammatory signals mediated by CD4⁺ T cells are each necessary to achieve the

airway epithelial mucus levels observed in OVA-treated wild-type mice.

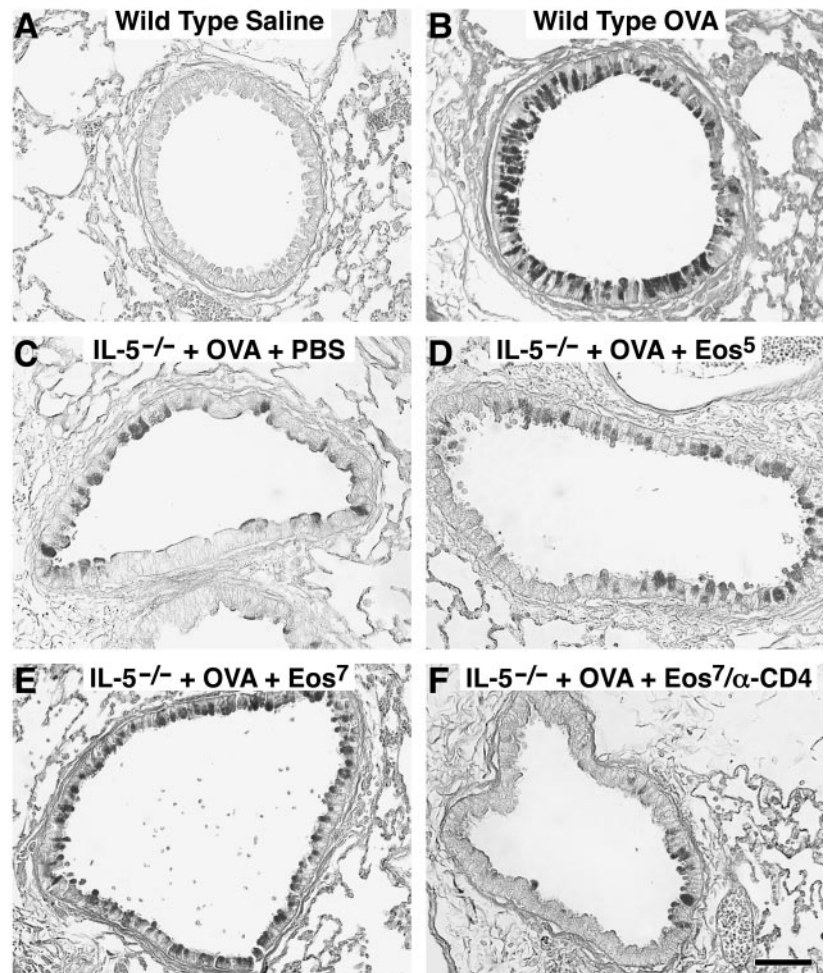
The AHR associated with OVA sensitization/aerosol challenge requires both eosinophils and CD4⁺ T cells

The potential role(s) of eosinophils in the development of OVA-induced AHR to methacholine provocation was assessed by whole-body plethysmography. The resulting dose response curves (means of single-animal measurements) are presented in Fig. 8. These data compare the responses of wild-type and IL-5^{-/-} mice with responses occurring in IL-5^{-/-} mice following adoptive transfer of eosinophils. The dose response curves confirm earlier studies (see, for example, Ref. 8) and demonstrate the loss of AHR in OVA-treated IL-5^{-/-} mice (i.e., no significant differences were observed in the response of OVA-treated IL-5^{-/-} mice and either saline-treated wild-type or IL-5^{-/-} animals). However, this pathophysiologic response was recovered in OVA-treated IL-5^{-/-} mice following the restitution of the pulmonary eosinophilia by adoptive transfer. The recovery of AHR was restricted to recipient mice that were OVA sensitized/challenged and did not occur in saline-treated IL-5^{-/-} animals (i.e., the presence of pulmonary eosinophils is necessary but not sufficient to induce AHR). In addition, the recovery of AHR was restricted to OVA-treated mice in which the pulmonary eosinophilia was restored to OVA-treated wild-type levels and did not occur following partial restitution of the eosinophilia (Fig. 9). The concurrent administration of anti-IL-5 Ab had no measurable effect on the AHR that occurs in OVA-treated IL-5^{-/-} mice following eosinophil transfer (Fig. 9), demonstrating that the observed pathophysiology is not a consequence of reconstituting IL-5 in these animals. The data of Fig. 9 also demonstrate that similar to the induction of OVA-mediated intracellular airway epithelial mucus accumulation, the recovery of AHR following adoptive transfer of eosinophils did not occur in OVA-treated IL-5^{-/-} mice depleted of CD4⁺ T cells thus showing that both eosinophils and CD4⁺ T cells are each necessary, but alone insufficient, to achieve AHR in this model of allergen-mediated respiratory inflammation.

Discussion

The hypothesis that allergic respiratory pathology is a concomitant response of the induced pulmonary eosinophilia is based on a large

FIGURE 5. Pulmonary eosinophils and CD4⁺ T cells are each required to achieve the induced airway mucus accumulation observed in OVA-treated wild-type mice. Lung sections from formalin-fixed/paraffin-embedded tissue were stained for the presence of mucin with periodic acid-Schiff's reagent (counterstain: hematoxylin/methyl green). The photographs presented show representative examples of the staining that occurs in bronchioles. Airways from saline control (A) and OVA sensitized/challenged wild-type mice (B) are shown in comparison to similar airways from OVA sensitized/challenged IL-5^{-/-} mice following IT instillation of PBS (C) and OVA sensitized/challenged IL-5^{-/-} mice following IT instillation of 4 × 10⁵ eosinophils (Eos⁵) (D) or 4 × 10⁷ eosinophils (Eos⁷) (E). F, OVA-treated IL-5^{-/-} mice concurrently administered GK1.5 to deplete CD4⁺ T cells before IT instillation of eosinophils (IL-5^{-/-} + OVA + Eos⁷/α-CD4). Bar = 50 μm.



body of correlative (i.e., circumstantial) data from both asthma patients and mouse models. However, the advent of a strategy to specifically introduce eosinophils into the lungs of otherwise eosino-

phil-deficient mice now provides evidence that the allergen-induced eosinophilia is indeed causatively linked to the development of pulmonary pathology. In particular, eosinophil effector functions in the

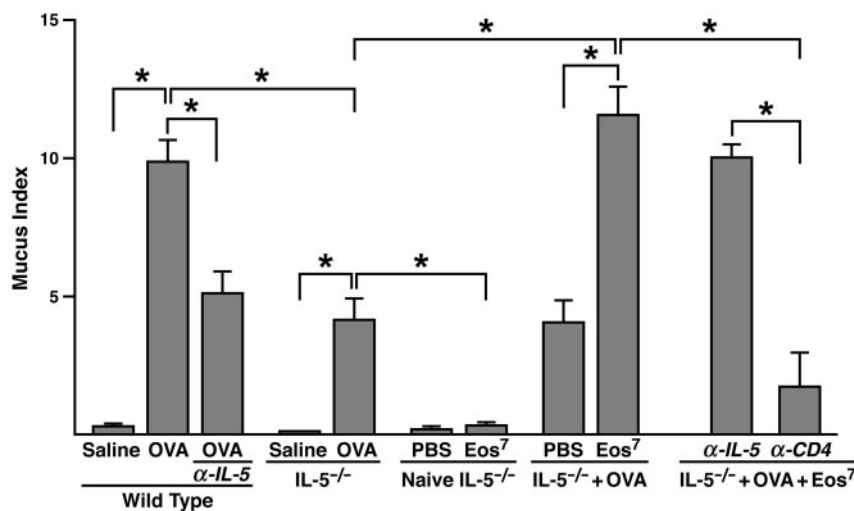


FIGURE 6. Restitution of the pulmonary eosinophilia in OVA sensitized/challenged IL-5^{-/-} mice restores intracellular airway epithelial mucus accumulation to levels observed in OVA-treated wild-type mice. Mucus accumulation (i.e., Mucus Index) was determined in groups of wild-type and IL-5^{-/-} mice following saline or OVA challenge vs naive and OVA-treated IL-5^{-/-} mice following IT instillation of 4 × 10⁷ eosinophils (Eos⁷) or PBS vehicle control. Control groups shown include OVA-treated wild-type mice administered anti-IL-5 mAb TRFK-5 (OVA/α-IL-5), OVA-treated IL-5^{-/-} mice concurrently administered TRFK-5 before IT instillation of eosinophils (IL-5^{-/-} + OVA + Eos⁷/α-IL-5), and OVA-treated IL-5^{-/-} mice concurrently administered GK1.5 to deplete CD4⁺ T cells before IT instillation of eosinophils (IL-5^{-/-} + OVA + Eos⁷/α-CD4). All evaluations of mucus content were performed in duplicate as independent observer-blinded assessments of five animals per group (two to five sections per animal). *, *p* < 0.05.

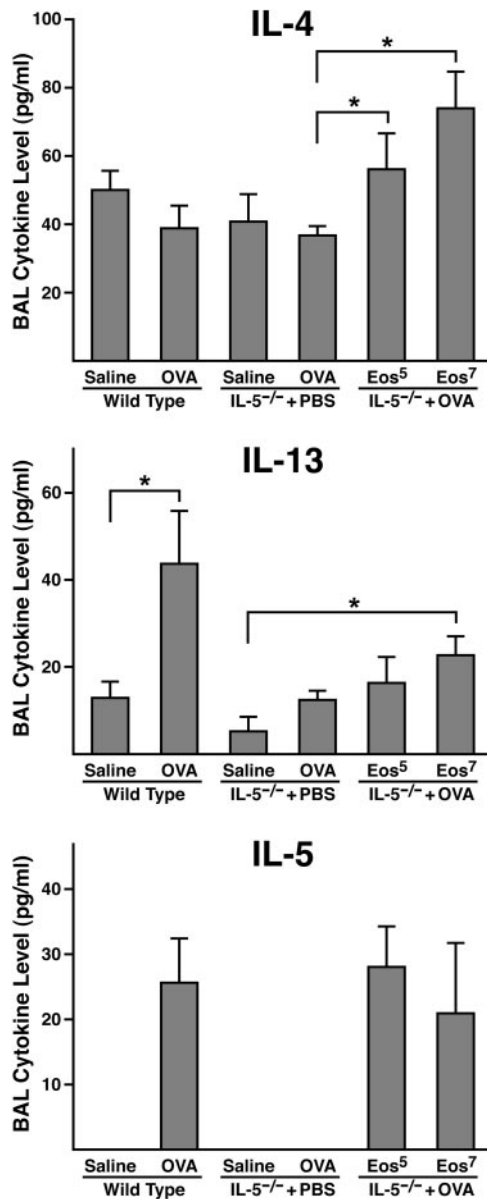


FIGURE 7. Adoptive transfer of eosinophils to the lungs of OVA sensitized/challenged IL-5^{-/-} mice results in the appearance of airway IL-5 and increases in BAL IL-4 and IL-13 levels. Cohorts of mice ($n = 5$) were sacrificed on day 28 of the OVA protocol to determine BAL levels of IL-4, IL-5, and IL-13 in wild-type animals following saline or OVA challenge, saline and OVA challenged IL-5^{-/-} mice following IT instillation of PBS, and OVA-treated IL-5^{-/-} mice following IT instillation of either 4×10^5 (Eos⁵) or 4×10^7 (Eos⁷) eosinophils. Data represent means \pm SE. *, $p < 0.05$.

lung are necessary for allergen-induced airway mucus accumulation and the development of AHR in response to a cholinergic receptor agonist.

The IT instillation of peripheral blood eosinophils into the lungs of IL-5^{-/-} mice highlights the ability of these leukocytes to engage in reciprocal movement from the airway lumen and lung tissue. Specifically, eosinophils in the airway lumen are capable of traversing the respiratory epithelium. This phenomenon was noted earlier in an elegant study by Shi et al. (35) who demonstrated that eosinophils are capable of trafficking from the lumen to draining pulmonary lymph nodes where they function as APC and activate T cells. The physiological relevance of this APC function remains

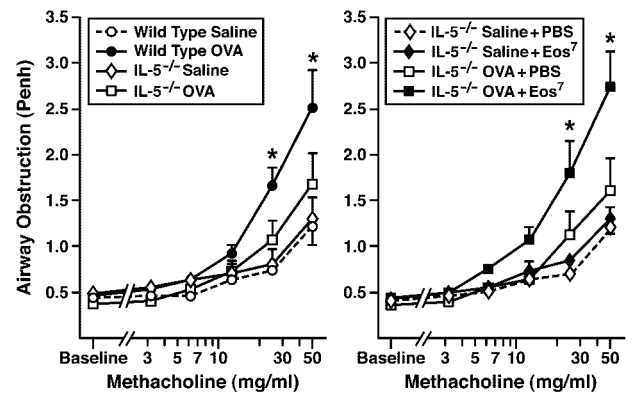


FIGURE 8. Pulmonary eosinophils are necessary but not sufficient to induce allergen-mediated AHR. Methacholine dose-response curves result from group mean data (\pm SE) of 10–12 mice/group. Airway reactivity (Penh) in response to increasing doses of nebulized methacholine was assessed by whole-body plethysmography. The data are presented side by side in two separate plots for clarity: saline vs OVA treatment of wild-type and IL-5^{-/-} mice and saline vs OVA treated IL-5^{-/-} mice administered either 4×10^7 eosinophils (Eos⁷) or PBS vehicle control. *, $p < 0.05$, (wild-type OVA) vs (wild-type saline) and (IL-5^{-/-} OVA + Eos⁷) vs (IL-5^{-/-} OVA + PBS).

to be established; however, the ability of eosinophils to traverse the airway epithelium may have a logistical advantage for host defense. The differential movement of eosinophils to the lumen may permit these cells to encounter pathogens before they violate the

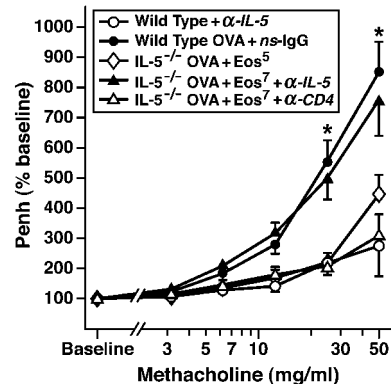


FIGURE 9. Recovery of allergen-induced AHR in IL-5^{-/-} mice following eosinophil adoptive transfer is dependent on the extent to which the pulmonary eosinophilia is restored as well as the presence of CD4⁺ cells. Airway reactivity (percent baseline Penh) in response to increasing doses of nebulized methacholine was assessed by whole-body plethysmography ($n = 10$ –12 mice/group). The groups examined include: wild-type OVA-treated mice concurrently administered either nonspecific IgG (*ns*-IgG) or TRFK-5 (α IL-5), OVA-treated IL-5^{-/-} mice following IT instillation of 4×10^5 (Eos⁵) eosinophils (IL-5^{-/-} OVA + Eos⁵), and OVA-treated IL-5^{-/-} mice concurrently administered either TRFK-5 or GK1.5 before IT instillation of 4×10^7 (Eos⁷) eosinophils (IL-5^{-/-} OVA + Eos⁷ + α IL-5 and IL-5^{-/-} OVA + Eos⁷ + α CD4, respectively). *, $p < 0.05$, (wild-type OVA + *ns*-IgG) and (IL-5^{-/-} OVA + Eos⁷ + α IL-5) vs the remaining three groups. The baseline Penh values (\pm SE) of the animal groups examined are: wild-type OVA-treated mice administered *ns*-IgG, 0.30 ± 0.01 ; wild-type OVA-treated mice administered anti-IL-5, 0.33 ± 0.01 ; IL-5^{-/-} OVA-treated mice following IT instillation with 4×10^5 eosinophils, 0.39 ± 0.08 ; IL-5^{-/-} OVA-treated mice administered anti-IL-5 following IT instillation with 4×10^7 eosinophils, 0.33 ± 0.04 ; IL-5^{-/-} OVA-treated mice administered α CD4 following IT instillation with 4×10^7 eosinophils, 0.34 ± 0.02 .

integrity of the airway epithelium. Presumably these interactions are part of strategies to kill and/or expel these invading microorganisms, and in doing so, eosinophils would be able to ferry Ags back to sites of T cell maturation (i.e., pulmonary lymph nodes), enhancing acquired immune responses. This model does not prevent the reciprocal movement of eosinophils (i.e., from the interstitium to the lumen and then back to the interstitium) from also having an additional functional significance. For example, the execution of eosinophil effector functions may be linked to this reciprocal movement. That is, eosinophils which traffic to the airway lumen in response to allergen-mediated inflammation would receive signals and/or interactions in this milieu that elicit activation, resulting in their subsequent recruitment back to the lung interstitium/pulmonary lymphatic circulation, and ultimately the execution of effector functions leading to pulmonary pathology.

The eosinophil dependence of allergen-induced AHR and airway mucus accumulation suggests that effector functions have multiple effects in the lung which are physiologically relevant. The link between eosinophils and AHR, in particular, has been difficult to establish as studies abound both supporting and opposing the null hypothesis. Studies which demonstrate that AHR occurs in the absence of a significant airways eosinophilia (see, for example, Refs. 13–17) have been uniquely problematic. However, these studies have a significant limitation, they define causation of pathologies as independent of eosinophils because in some model systems the pathologies occur in the absence of this cell. An alternative explanation is that AHR is a complex response resulting from multiple, and potentially independent, mechanisms. Thus, eosinophil-dependent and -independent pathways leading to AHR likely exist and thus under some experimental conditions AHR may still occur in the absence of this cell. The conflict in the literature regarding the background strain dependency of AHR in IL-5^{-/-} mice (Ref. 8 vs Ref. 16), and, in turn, the unique contribution of eosinophils to AHR, may be a consequence of the differential importance of these multiple pathways in various strains of mice (i.e., eosinophils contribute to AHR in all strains but in some strains eosinophil dependent pathways are prominent whereas in others they are not).

The logistics surrounding eosinophils and airway mucus accumulation are clearly more complex as the development of this histopathology appears unrelated to the presence or absence of pulmonary eosinophils (11, 36). In fact, many studies have suggested that airway mucus accumulation is linked only with CD4⁺ T cells and not eosinophils (see, for example, Ref. 11). However, the hegemony of CD4⁺ T cells may have obscured the quantitative dependence of airway mucus production on the presence of eosinophils. These previous studies showed that airway mucus production was possible in the absence of eosinophils, but did not demonstrate that eosinophils have no role in this histopathology. Indeed, the conclusion that eosinophils quantitatively contribute to allergen-mediated mucus production is consistent with the observation that although mucus production still occurred in OVA-treated IL-5^{-/-} mice, it occurs at levels significantly lower than either OVA-treated wild-type mice or OVA-treated IL-5^{-/-} animals following adoptive transfer of eosinophils. These observations imply that multiple pathways contribute to goblet cell metaplasia/mucus production following allergen provocation, including eosinophil-independent and -dependent pathways. It is noteworthy that potential mechanisms of eosinophil-dependent contributions to mucus production, as well as eosinophil-dependent AHR, are not exclusive of T cell mediated activities. This observation leaves open the possibilities that T cells elicit eosinophil effector functions which contribute to pulmonary pathologies or that eosinophils induce additional T cell-mediated activities augmenting pathology. The ob-

served increase in BAL Th2 cytokine levels following eosinophil transfer into OVA-treated IL-5^{-/-} recipients may be representative of either mechanism as eosinophils themselves have the potential to elaborate Th2 cytokine levels (37) as well as the ability to regulate Th2 cytokine (e.g., IL-13) production by T cells (38). These effects, particularly within localized microenvironments within the lung, therefore have the potential to induce/enhance mucus production (as well as AHR (39)) in such a way as to give the appearance that these phenomena are elicited by T cells alone.

Several important insights regarding eosinophil activities and the onset/progression of pulmonary pathologies are apparent, and worth noting, despite the unresolved issues surrounding specific effector functions: 1) Eosinophils alone are insufficient to elicit either mucus accumulation or AHR (i.e., transfer of eosinophils into naive IL-5^{-/-} had no effect on either pathology), suggesting that the inflammatory process provides one or more signals leading to the execution of eosinophil effector functions. 2) The loss of IL-5 activities in OVA-treated IL-5^{-/-} mice, which resulted in both a significant decrease in mucus accumulation and AHR, were recoverable (i.e., equivalent to wild-type levels) following adoptive transfer of eosinophils (Note: the effects on AHR are likely also to be quantitative and not absolute, but the small size of the changes, and the methodology used, preclude statistically significant assessments). Thus, IL-5 activities appear to be restricted to effects on eosinophils in this mouse model and not the additional cell types that have been implicated as potential targets of IL-5 (e.g., B cells (20), T cells (7, 22, 23), and airway smooth muscle (24)). Further support for this conclusion is provided by our demonstration that the selective ablation of eosinophils in the lung, without concurrent effects on T cell activities, also leads to the attenuation of airway mucus and the loss of AHR (40). The specific function(s) of IL-5 on eosinophils remain unresolved as these effects may be a consequence of either an IL-5 proliferative capacity inducing pulmonary eosinophilia, a regulative capacity necessary for eosinophil activation, or the additional possibility that IL-5 is required for the recruitment of these leukocytes from circulation (6). 3) Potential contributions of eosinophils to pulmonary pathologies were dependent on the presence of CD4⁺ T cells. This phenomenon has also recently been described in a study showing that eosinophil associated lung tissue damage in a helminth infestation model system was dependent on the presence of CD4⁺ T cells (41). Thus, it appears that signaling between these cells (either directly or through third party intermediates) is necessary for the activation of one or both cell types, and the execution of eosinophil effector functions *in vivo*. This signaling may occur through direct cell-cell interactions or *in trans* through the release of soluble factors. The demonstrated APC function of eosinophils provides a mechanism by which direct cell-cell interactions may lead to eosinophil activation. Significantly, these interactions are not necessarily limited to MHC-TCR mediated events. The demonstration that eosinophils also express costimulatory cell-surface receptors, such as CD28 and CD86 (42), suggests that multiple receptor-ligand interactions are possible between eosinophils and T cells that may result in signaling cascades in one or both cell types that ultimately leads to the recruitment, activation, and/or the execution of eosinophil effector functions. However, this cell-cell interaction paradigm requires physical contact between T cells and eosinophils. An alternative possibility, with potentially less restrictions, is a model in which interactions with eosinophils are mediated by a soluble factor secreted by T cells or a third party cell. Pulmonary basophils/mast cells may represent a potential third party cell. However, the observation that the restitution of the pulmonary eosinophilia in OVA-treated IL-5^{-/-} mice was not accompanied by significant changes in the number, location, or histological morphology of

pulmonary basophil/mast cells (data not shown) suggests that this unlikely although the specific role(s) of these cells remains to be defined. In contrast, many other potential T cell-dependent signals exist that are known to have effects on eosinophil activities, including the expression of IL-4/IL-13 (38, 43), small molecule lipid intermediates (e.g., leukotrienes or PAF (44–46)), and CCR3 chemokine ligands such as eotaxin (reviewed in Ref. 47). Each of these mechanisms also need not be mutually exclusive, and are all likely to occur as part of allergen-mediated inflammatory responses in the lung. Regardless of the eventual identification of the mechanism(s) mediating this interaction and the definition of specific eosinophil effector functions, the data support an expanded view of eosinophil activities in the lung and suggest that this cell is part of a pathway(s) underlying allergic respiratory inflammation and lung dysfunction.

Acknowledgments

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