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Eosinophils Promote Allergic Disease of the Lung by Regulating CD4$^+$ Th2 Lymphocyte Function[1]

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Eosinophils are primarily thought of as terminal effectors of allergic responses and of parasite elimination. However, limited studies suggest a more discrete immunomodulatory role for this leukocyte during these inflammatory responses. In this investigation, we highlight the potential of eosinophils to act as APCs and thus modulators of allergic responses by influencing Th2 cell function. In response to Ag provocation of the allergic lung, eosinophils rapidly trafficked to sites of Ag deposition (airways lumen) and presentation (lung-associated lymph nodes and T cell-rich paracortical zones). Eosinophils from the allergic lung expressed class II MHC peptides, T cell costimulatory molecules (CD80 and CD86), and rapidly internalized and processed Ag that was sampled from within the airway lumen. Ag-loaded eosinophils promoted the production of IL-4, IL-5, and IL-13 in cocultures with in vitro-polarized Th2 cells and induced IL-5 production in a dose-dependent manner from Ag-specific CD4$^+$ T cells isolated from allergic mice. In addition, Ag-loaded eosinophils primed for Th2 cell-driven allergic disease of the lung when transferred to naive mice. Thus, eosinophils have the potential to not only activate Th2 cells to release disease-modulating cytokines but also to assist in priming the immune system for allergic responses. This investigation highlights the potential of eosinophils to not only act as terminal effector cells but also to actively modulate allergic inflammation by amplifying Th2 cell responses. ***The Journal of Immunology,*** **2001, 167: 3146–3155.**

The eosinophilic leukocyte is a predominant feature of immunological responses associated with allergic diseases of the lung, skin, and gastrointestinal tract and in helminth infection (1, 2). At sites of allergic disease and parasitic infection, eosinophils are thought to occupy key roles in pathogenesis and worm expulsion through the release of proinflammatory molecules and granular proteins (1, 2). Although the eosinophil is classically thought to act as an effector cell, there is emerging in vivo evidence that this leukocyte may also have discrete afferent immunomodulatory roles.

Eosinophils express MHC class I (3) and II (4–9) molecules and the costimulatory molecules CD28 (10), CD40 (11, 12), CD80, and CD86 (10, 13), suggesting that these cells can directly communicate with helper and cytolytic T cells to regulate humoral and viral immune responses. In addition, eosinophils also secrete a range of cytokines that are not only proinflammatory but also function as growth factors, stimulants, and chemoattractants (e.g., IL-2, IL-4, IL-5, IL-10, IFN-γ, IL-12, RANTES, and IL-16) for T cells (14–23). Notably, IL-4 and IFN-γ are critical factors for the polarization of Th lymphocytes (Th cells) to the Th2 and Th1 phenotypes, respectively (24, 25). In a limited number of in vitro investigations, evidence for communication between eosinophils and T cells has been demonstrated (26). In particular, eosinophils have been shown to stimulate the proliferation of cultured Ag-specific T cells (7, 27, 28). In vivo evidence of an immunomodulatory role for this granulocyte is also emerging. Through the early production of IL-4, eosinophils have been shown to play an important role in the inductive phase of the immune response to *Schistosoma mansoni* egg infestation and potentially in the development of egg-specific Th2 cells (29). Recruitment of eosinophils to the airways of naive mice by transient expression, by viral vectors of either GM-CSF or both IL-5 and eotaxin, promotes T cell-mediated features of allergy after Ag (OVA) inhalation (30, 31). Recently, Ag-loaded eosinophils instilled intratracheally into naive mice were shown to migrate into local lymph nodes and localize to the T cell-rich paracortical zones where they stimulated expansion of CD4$^+$ T cells (9). Mast cells and eosinophils have also been shown to traffic from the jejunum to the spleen and draining lymph nodes after helminth infection (32). Thus, an emerging concept is that within the correct microenvironment granulocytes such as eosinophils and mast cells may communicate with T cells, providing mechanisms for interactions between the innate and adaptive immune responses.

Large numbers of eosinophils are recruited to mucosal surfaces in response to allergen provocation in allergic diseases associated with the respiratory (asthma and rhinitis) and gastrointestinal (food allergy) tracts (33, 34). These recruitment sites are often environments rich in cytokines and chemokines where eosinophils may become activated and potentially participate in the regulation of the local immune response. Notably, tissue dwelling, but not circulating eosinophils, in allergic individuals express HLA-DR (4, 5) and this phenotypic difference suggests a functional role for recruited eosinophils in local T cell-regulated processes. Collectively, the above investigations are indicative of a modulatory role for eosinophils in immune processes associated with the afferent arm of allergic diseases and parasitic infection. However, it is not known whether eosinophils can prime or amplify the immune system for an allergic response or liberate cytokines from T cells.

In this investigation, we show that in response to allergen provocation of the airways, eosinophils not only accumulate in the blood and airways of allergic mice, but also localize rapidly to

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lung-associated lymph nodes (LALN).[3] Furthermore, we demonstrate the ability of luminal airway eosinophils to take up and process exogenous Ag, to localize in the allergic lung and LALN tissue, and that in the presence of Ag, eosinophils not only induce the proliferation of $CD4^+$ T cells but also promote secretion of Th2 cytokines. Moreover, we report the ability of Ag-loaded eosinophils to induce Th2-mediated allergic disease of the lung when transferred to naive recipients. These data further highlight the multipotential role of the eosinophil in immunological processes not only associated with the expression but also the early phases of the development of allergic disease of the lung.

Materials and Methods

Mice

C57BL/6, BALB/c, and strain-matched IL-5-transgenic mice (Ref. 35 and L. A. Dent, unpublished observations) (the latter with ~49 transgenes copies, male, 6–8 wk old, and backcrossed to the 12th generation at the University of Adelaide, Adelaide, Australia) were supplied by the pathogen-free facility at the John Curtin School of Medical Research, Australian National University (Canberra, Australia). Mice were treated according to Australian National University Animal Welfare Guidelines.

Purification of eosinophils from the airways of allergic mice

C57BL/6 mice were sensitized by i.p. injection with 50 μg OVA/1 mg Alhydrogel (Commonwealth Serum Laboratories, Parkville, Australia) in 0.9% sterile saline on days 0 and 12. On days 24, 26, 28, and 30, the mice were aeroallergen (OVA, 10 mg/ml; total aerosol dose, 50 mg) challenged three times for 30-min periods (at 30-min intervals; allergic C57BL/6). The aerosol was generated by a RapidFlo nebulizer bowl (Allersearch, Melbourne, Australia) with an airflow rate >10 L/min to produce particles of 2 μm in diameter. Twenty-four hours after the last aeroallergen challenge, mice were sacrificed by cervical dislocation and their airways were lavaged (36). Eosinophils were purified from bronchoalveolar lavage fluid (BALF) by flow cytometry using forward vs side scatter and polarization of light (37). The purity of the enriched population of eosinophils was ≥98% as determined by differential staining of cytospins with Giemsa-May-Grünwald.

Visualization of eosinophils in airways tissue and LALN by light and electron microscopy

Specimens of lung or LALN were fixed in 10% phosphate-buffered Formalin, sectioned, and stained with Lendrum's carbolchromotrope stain (38). Leukocytes were identified by morphological criteria and quantified. For transmission electron microscopy, LALN and associated lymphatic ducts were immersed in a fixative consisting of 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The specimens were fixed overnight and then postfixed in 1% osmium tetroxide for 90 min. Specimens were then dehydrated in graded acetone solutions and embedded in Spur's Resin (ProSciTech, Sydney, Australia). For high-resolution light microscopy, semithin (1-μm) sections of the embedded tissue were cut and stained with toluidine blue. These sections were used to select areas of interest for electron microscopic examination. Ultrathin sections (80–85 nm) were cut on a Reichert-Jung Ultracut E ultramicrotome and routinely contrasted with uranyl acetate and lead citrate. The sections were examined using a Hitachi 7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Monitoring eosinophil migration into and from BALF and LALN during allergic inflammation

To measure eosinophil migration into BALF and LALN in response to Ag provocation, mice were sensitized (as described above for allergic C57BL/6) and exposed to an aerosol of OVA (10 mg/ml) for 30 min on days 24–26. BALF and LALN were analyzed at 0, 3, 6, 9, 24, 48, and 72 h after the first aerosol (received on day 24). This Ag delivery regimen allowed analysis of the spatial and temporal aspects of eosinophil recruitment to these compartments in the early phase of the allergic response when changes in lymph node diameter are negligible. Eosinophils per high-powered field (HPF) were counted in histological sections (×40 objective). Eosinophils in BALF were determined by differential staining of cytospins with Giemsa-May-Grünwald. To measure eosinophil clearance from BALF and LALN, samples were taken from sensitized and aerosolized mice (as described above for allergic C57BL/6) at 3, 6, 9, 12, 15, and 18 days after the last aerosol challenge. This Ag delivery regimen induced peak eosinophil recruitment to BALF and LALN and maximal changes in lymph node diameter by day 31, providing a model to investigate resolution of eosinophilia from these compartments. As a control, brachial lymph nodes were also sampled at all time points to compare eosinophil trafficking in extrapulmonary lymphoid tissue.

Fluorescent labeling of eosinophils and analysis of trafficking in vivo

Eosinophils were taken from the peritoneal cavity of C57BL/6 IL-5-transgenic mice and FACS sorted (98% purity) before in vitro staining with the nuclear dye Hoechst 33342 (Molecular Probes, Eugene, OR). After washing and resuspension in PBS, stained cells (4×10^6/mouse, 90% labeled) were i.v. isolated and injected into OVA-sensitized mice after the third aerosol challenge (as described above for allergic C57BL/6). At 2 and 12 h posttransfer, blood and tissue samples (BALF, lung, spleen, and LALN) were taken after cardiac perfusion with PBS, and the number of fluorescent eosinophils trafficking to these compartments were determined after digestion of samples. Fluorescent cells were detected with a BD Biosciences FACScan flow cytometer (San Jose, CA) in conjunction with CellQuest (BD Biosciences) and WinMDI software packages (kindly provided by J. Trotter, The Scripps Research Institute, La Jolla, CA).

Eosinophil internalization and processing of OVA in vitro and in vivo

To demonstrate cellular internalization and processing of OVA in vitro, eosinophils were purified from the peritoneal exudates of IL-5-transgenic mice and incubated with 1 mg/ml OVA or DQ OVA (Molecular Probes) in 1 ml of HBSS for 30 or 240 min at 37°C. Cells were then washed three times in 10 ml of PBS before fixing in 4% paraformaldehyde in PBS. Analysis of fixed cells was performed using FACScan with WinMDI software. Eosinophil-gated regions were determined based on forward and side scatter signals and fluorescent cells ($DQ\ OVA^+$) detected in the FL-1 channel were compared with cells incubated with native OVA.

To demonstrate cellular internalization and processing of OVA in vivo, mice sensitized and aerosoled with OVA (as described above for allergic C57BL/6) were given intranasal doses of 1 mg of DQ OVA in HBSS after each aerosol challenge. The DQ OVA challenges were done in this manner to directly coincide with each individual aerosol. Twenty-four hours after the last aerosol/DQ OVA challenge, mice were sacrificed by cervical dislocation and their airways were lavaged. BALF cells were analyzed using a FACScan to identify fluorescent ($DQ\ OVA^+$) eosinophil populations.

Measuring expression of CD80, CD86, and class II MHC on eosinophils from allergic lung

Eosinophils (10^5 cells) purified from BALF samples taken from allergic C57BL/6 mice were incubated with PE-conjugated anti-mouse CD80 or PE-conjugated anti-mouse CD86 (BD PharMingen, San Diego, CA) for 30 min on ice before FCS underlay to remove dead cells. Cells were then centrifuged at 500 × *g* for 5 min at 4°C. The supernatant was removed and the cell pellet resuspended in HBSS, on ice, before analysis by flow cytometry. To measure MHC class II, eosinophils were purified from the BALF of allergic BALB/c mice and incubated with biotinylated anti-I-A/I-E Ab (M5/114), before washing and incubation with streptavidin-PE or with FITC-conjugated conformation-dependent anti-I-A (MKD6). Allergic airways inflammation was induced and eosinophils were isolated from the BALF of BALB/c mice (as described above for allergic C57BL/6). Analysis of fluorescence was performed using FACScan and WinMDI.

In vitro generation of OVA-specific Th2 T cells

OVA-specific $CD4^+$ T cells were derived from BALB/c mice (6–8 wk of age) sensitized by i.p. injection with 50 μg OVA/1 mg in 0.9% sterile saline. Six days after sensitization, donor mice were sacrificed by cervical dislocation, the spleens were excised, and the splenocytes were disaggregated. Erythrocytes were lysed, and the washed splenocytes were resuspended at 5×10^6 cells/ml in complete tissue culture medium consisting of HL-1 (BioWhittaker, Walkersville, MD) with 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 mg/L neomycin sulfate. Splenocytes were then cultured for 4 days at 37°C in the presence of 200 μg/ml OVA, recombinant murine IL-4 (20 ng/ml), and anti-IFN-γ Ab (R46A2, 40 μg/ml) to generate Th2 cells. $CD4^+$ Th2 cells were then isolated using high-gradient magnetic MiniMACS separation columns (MACS separation; Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously (39). The purity of the enriched $CD4^+$ T cell fraction was uniformly above 96% as

[3] Abbreviations used in this paper: LALN, lung-associated lymph node; BALF, bronchoalveolar lavage fluid; HPF, high-powered field.

determined by flow cytometry (result not shown). Purified $CD4^+$ T cell populations were analyzed for Ag-specific cytokine production (IL-4, IL-5, IL-13, and IFN-γ) and intracellular cytokine profiles were determined to confirm in vitro polarization to the Th2 cells (results not shown).

Eosinophils as APCs to OVA-specific $CD4^+$ Th2 cells generated in vitro

To determine the ability of eosinophils to act as APCs and stimulate cytokine production from Th2 cells, these leukocytes were cocultured with and without eosinophils. Eosinophils that were freshly isolated from the peritoneal cavities of BALB/c IL-5-transgenic mice were purified and cultured in equal numbers with Th2 cells (5×10^5 cells/well) in complete medium in the presence of 200 μg/ml OVA in 96-well plates (250 μl/well). For comparison, Th2 cells (5×10^5 cells/well) were also cultured under the same conditions with mitomycin C (25 μg/ml)-treated splenocytes (5×10^5 cells/well) (as a source of conventional APCs). Ninety-six hours later, supernatants from cultures were collected for cytokine analysis.

Eosinophils as APCs to OVA-specific $CD4^+$ T cells from allergic mice

In an independent experiment, $CD4^+$ T cells were purified from the spleens of allergic C57BL/6 mice using MACS separation as described above. $CD4^+$ T cells (5×10^5 cells/well) that were incubated with purified BALF eosinophils (3×10^4, 5×10^4, and 1×10^5 cells/well) in complete medium in the presence of 200 μg/ml OVA in 96-well plates. Cell-free culture supernatants were collected 96 h later and stored in aliquots at -70°C before analysis of IL-5. The $CD4^-$ fraction was also used as an alternative source of conventional APCs.

Cytokine analysis

IL-13 (R&D Systems, Minneapolis, MN), IL-4, IL-5, and IFN-γ (all from BD PharMingen) concentrations were determined in the supernatants from OVA-stimulated $CD4^+$ T cells by ELISA according to the manufacturer's protocol.

Induction of allergic airways inflammation in naive mice by adoptive transfer of eosinophils loaded exogenously with Ag

Eosinophils purified by flow cytometry (98% pure) from peritoneal washes of IL-5 C57BL/6-transgenic mice were incubated in HBSS containing 1 mg/ml OVA for 30 min at 37°C. Eosinophils were then washed (five times) in excess HBSS to remove unloaded OVA. Ag-loaded or Ag-free eosinophils (2×10^6) suspended in HBSS were then injected i.p. into naive C57BL/6 mice on days 0 and 9. Recipients were then exposed to OVA by aerosol on days 15, 17, 19, and 21. On day 22, blood, BALF, lung, and sera samples were taken for analysis. Blood, BALF, and lung samples were analyzed for the presence of inflammatory cells (36). Sera were analyzed for OVA-specific IgG1 Ab (39). LALN were also harvested, physically disaggregated, and isolated cells were cultured for 96 h at a density of 5×10^5 cells/well in MLC medium with or without 200 μg of OVA. Culture supernatants were then taken and assayed by ELISA for IL-5 and IL-13.

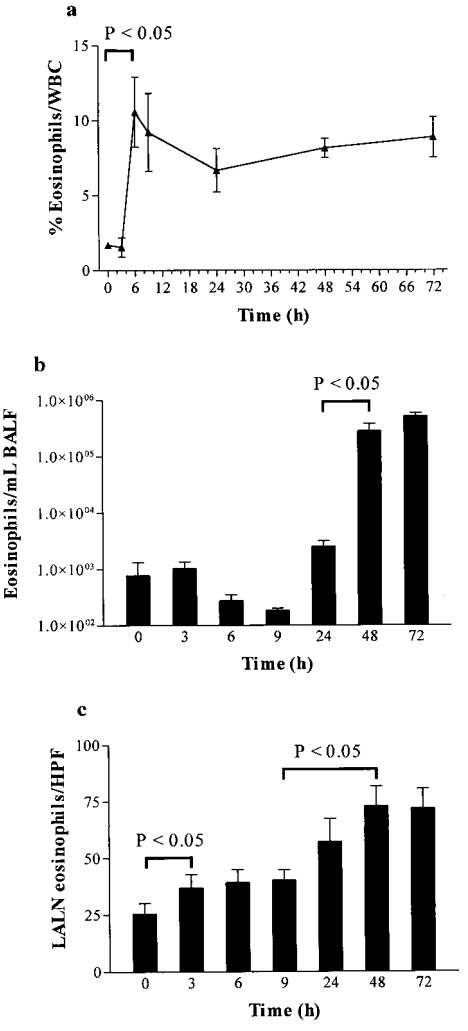

FIGURE 1. Eosinophil numbers in blood and pulmonary compartments after Ag provocation of allergic mice. Sensitized mice were challenged with a 30-min aerosol and samples were taken at the times shown. A significant rise in blood eosinophils (*a*) was observed at 6 h after the first aerosol and persisted for at least 72 h. Eosinophils appeared in BALF (*b*) and LALN (*c*) 48 h after initial aeroallergen challenge. Each time point represents hours lapsed since first aerosol exposure. Data represent mean ± SEM from groups of four mice. Significant differences in means were determined by Student's *t* test ($p < 0.05$).

Results

Eosinophils migrate to lung and LALN conjointly after aeroallergen challenge of sensitized mice

To determine the potential of eosinophils to act as APCs during allergic reactions in the lung, we examined the spatial and temporal aspects of eosinophil trafficking to regions of Ag deposition (airways lumen) and presentation (LALN) in sensitized mice after aeroallergen challenge. OVA was delivered to the airways over a period of 72 h and eosinophil numbers were characterized in the blood, BALF, and LALN (Fig. 1). Blood eosinophilia peaked 6 h after the first aerosol challenge and was sustained for at least 72 h (Fig. 1*a*). Significant numbers of eosinophils had trafficked to the BALF (Fig. 1*b*) and LALN (Fig. 1*c*) within 48 h of the initial exposure to OVA. Electron microscopy of LALN at 48 h showed eosinophils in the draining lymphatic duct and within the subcapsular sinus of the node (Fig. 2, *a* and *b*). In some sections, eosinophils were observed in close proximity to lymphocytes (Fig. 2*b*) and displayed characteristics of activation, including loss of granule matrix and crystalloid core. Although some cells showed loss of secondary granules, the majority showed no signs of degranulation nor condensation of nuclear chromatin indicative of the late stages of apoptosis. Collectively, these data show that eosinophils are not exclusively recruited to the airways during allergic responses but also migrate to regions where Ag is deposited (the airway lumen) and those associated with Ag-specific T cell stimulation and expansion (LALN). However, whether eosinophils traffic from the airways lumen to the LALN was not determined.

Eosinophil numbers in LALN, lung, and BALF return to basal levels 20 days after cessation of aeroallergen challenge

The kinetics of resolution of eosinophilia in various compartments of the lung during the refractive phase of allergic inflammation is poorly defined. To characterize the clearance of eosinophils, a strong aeroallergen challenge regimen was used to generate large numbers of eosinophils in pulmonary compartments and the blood of allergic mice. The BALF, lung tissue, LALN and blood were then characterized for 20 days after cessation of allergen exposure. Allergic mice were sacrificed every 3 days, beginning after the last

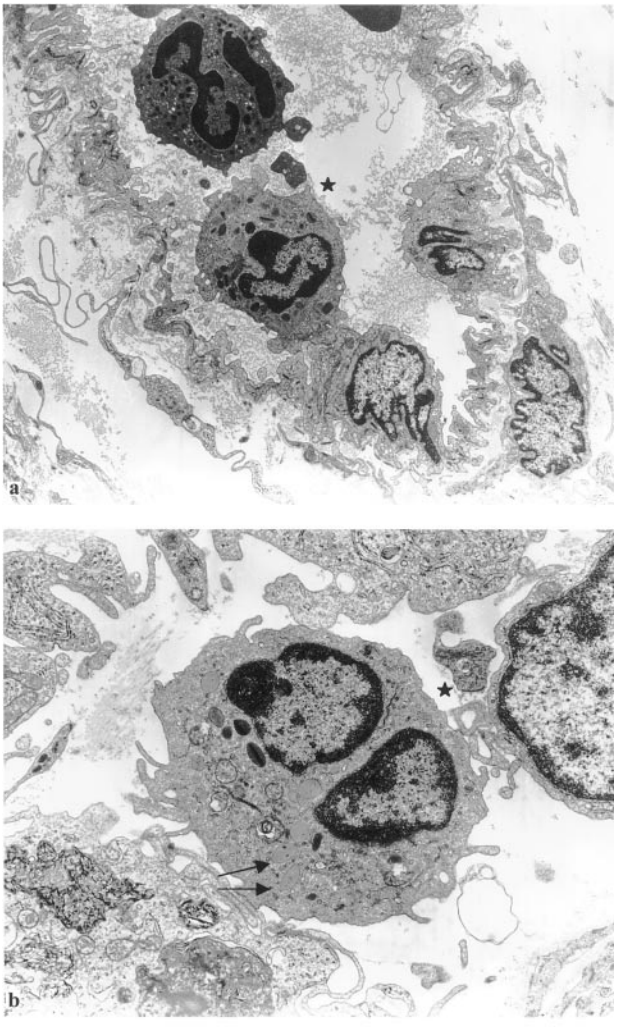

FIGURE 2. Eosinophils localize to LALN after Ag provocation of the allergic lung. Electron microscopy showed that eosinophils caught in transit to the draining lymphatic duct (*a*, ⋆) and within the subcapsular sinus region in LALN of allergic mice (*b*). Some evidence of activation was noted (*b*, arrows) by the loss of crystalloid core and matrix of eosinophil granules. One eosinophil (*b*) appears to be interacting with a lymphocyte, evidenced by extended processes (*b*, ⋆). However, loss of crystalloid core was not a widespread phenomenon.

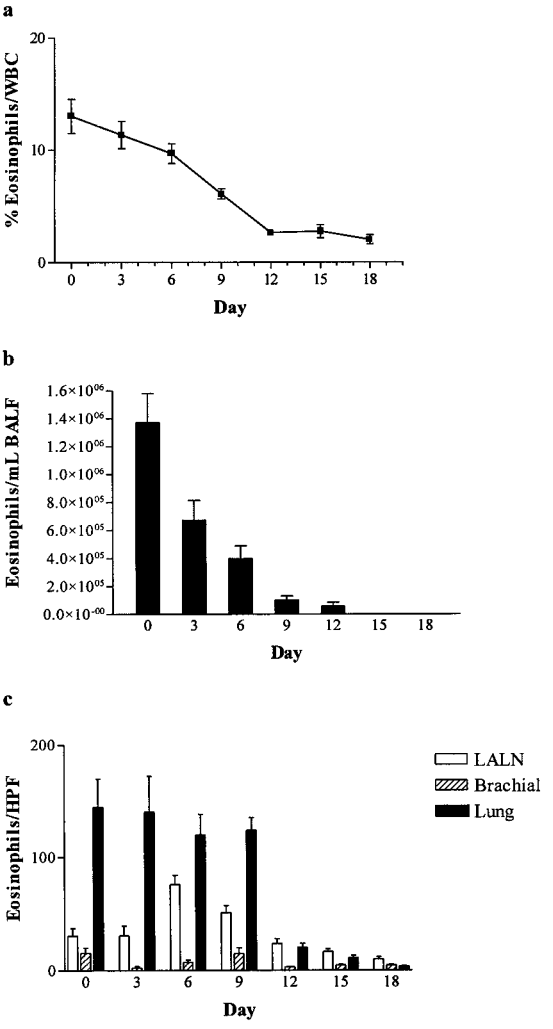

FIGURE 3. Resolution of eosinophilia from the blood and pulmonary compartments following Ag provocation of sensitized mice. Blood eosinophilia (*a*) gradually declined after cessation of Ag challenge, reaching basal levels by day 12. Eosinophil numbers in BALF (*b*) declined rapidly after cessation of aerosol and returned to baseline by day 15. Eosinophils per HPF in lung tissue (*c*) were maintained until 12 days after cessation of aerosol, whereas eosinophils in LALN did not peak until day 6, before gradually declining. Brachial lymph node eosinophil numbers did not change significantly.

aerosol challenge at day 31. Eosinophils/HPF for each tissue were counted and compared with residual numbers of eosinophils in the BALF and blood (Fig. 3). Brachial lymph nodes were also sampled for comparative purposes. Eosinophil numbers in the blood steadily declined over 12 days to baseline levels (Fig. 3*a*). BALF eosinophil numbers rapidly declined after cessation of allergen exposure (~50% within 3 days), returning to baseline levels by day 15 (Fig. 3*b*). By contrast, the number of eosinophils residing within the tissue (Fig. 3*c*) did not significantly decline until day 12, when the of pool eosinophils in the airways lumen was almost completely depleted (Fig. 3*b*). LALN eosinophil numbers continued to increase to day 6 after cessation of allergen exposure and then fell in parallel with tissue levels over the next 14 days to baseline levels (Fig. 3*c*). In all compartments eosinophil levels had returned to baseline within 18 days of the last allergen exposure. Eosinophil numbers in brachial lymph nodes did not change significantly in response to allergen provocation, suggesting that eosinophil trafficking was specific to events mediated within pulmonary lymph nodes and associated with allergic inflammation of the airways.

Eosinophils do not directly enter LALN from the blood following allergen provocation of the allergic lung

To further identify the migration pathway of eosinophils to pulmonary compartments during allergen provocation, 2×10^6 fluorescently labeled eosinophils were injected i.v. into allergic mice. Within 2 h of transfer, labeled cells were detected in blood and spleen (Fig. 4, *a* and *b*) and a significant population had trafficked to lung tissue but not to LALN (results not shown). By 20 h, labeled eosinophils were still migrating into the lung tissue; however, at this time fluorescent cells were not detected with in the LALN. These results suggest that eosinophils do not predominantly passage directly via the blood and high endothelial venules to LALN following allergen provocation but migrate via the pulmonary parenchyma.

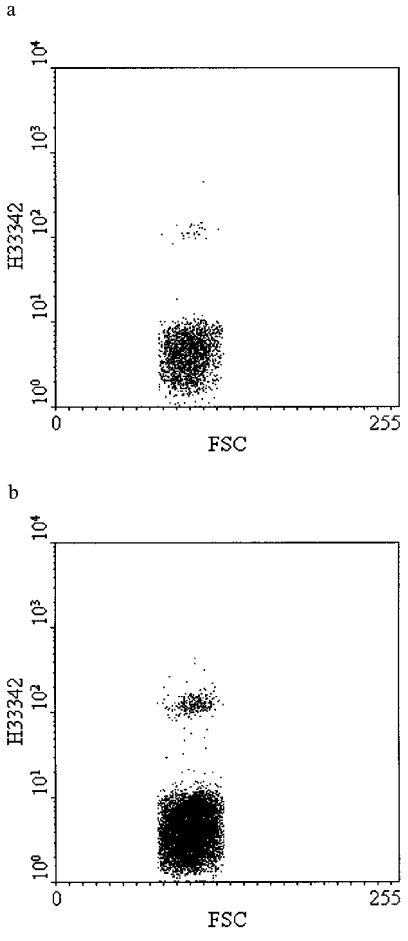

FIGURE 4. Eosinophils do not directly enter LALN from the blood following Ag provocation of the allergic lung. Eosinophils loaded with fluorescent dye were detected in blood (*upper panel*) and spleen (*lower panel*) at 2 h and in blood, spleen, and lung at 20 h (data not shown) following i.v. transfer to allergic mice. Fluorescent cells were not detected in LALN preparations. Cells were gated using characteristic forward and side scatter profiles of mouse blood eosinophils before analysis of fluorescence.

Eosinophils accumulate and process exogenous Ag both in vitro and in vivo

As eosinophils were found to localize at sites of allergen deposition (airways lumen) and in LALN of allergic mice following allergen challenge, we determined whether these cells could internalize and process exogenous Ag both in vitro and in the lumen of the allergic lung. Eosinophils were isolated from IL-5-transgenic mice and incubated with DQ OVA, a self-quenching fluorescently labeled OVA molecule, which is degraded to fluorescent peptides after uptake and intracellular processing (40). Eosinophils rapidly sequestered and processed DQ OVA (within 30 min) with maximal uptake and processing complete within 4 h of incubation (Fig. 5a). DQ OVA was also delivered intranasally to allergic mice after allergen provocation of the lung to determine the capacity of pulmonary eosinophils to accumulate and process exogenous Ag in the airways. A population of eosinophils in the BALF of these mice was found to incorporate and process DQ OVA (Fig. 5*b*). The varying degrees of fluorescence intensity of BALF eosinophils that were DQ OVA positive may be explained by the inability of all eosinophils to access the labeled Ag equally over the time of exposure and, thus, may reflect different rates of incorporation and processing of exogenous Ag in the allergic lung.

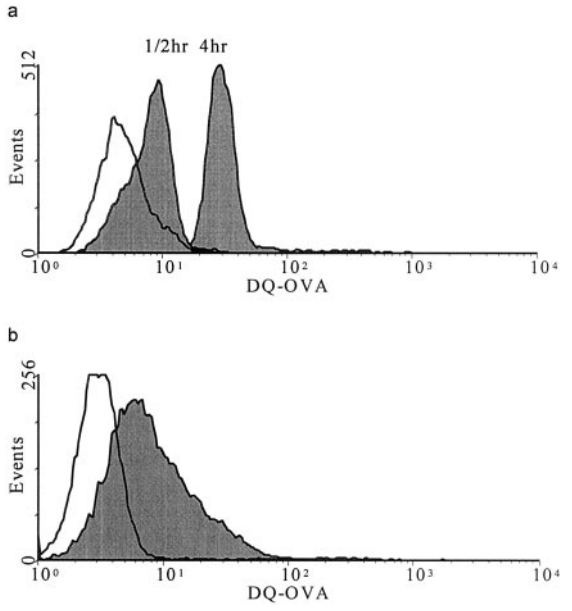

FIGURE 5. Eosinophils sequester and process exogenous Ag both in vitro and in the allergic lung. Purified IL-5-transgenic eosinophils (*a*, filled histogram) incubated in vitro with DQ OVA for 30 min and 4 h (indicated) show increased fluorescence after internalization and processing of DQ OVA. Fifty percent of BALF eosinophils (*b*, filled histogram) sequestered DQ OVA in vivo after intranasal administration. Significant fluorescence was not detected in mice that did not receive intranasal doses of DQ OVA (open histogram).

Eosinophils express class II MHC and costimulatory molecules in the allergic lung and stimulate cytokine production from Ag-specific CD4$^+$ T cells

We next determined whether eosinophils from the airways of allergic mice expressed molecules required for the presentation of exogenous Ags to T cells. Eosinophils purified from the BALF after allergen provocation expressed MHC class II and the T cell costimulatory molecules CD80 and CD86 (Fig. 6). BALF eosinophils expressed the costimulatory molecules CD80 (Fig. 6*a*) and CD86 (Fig. 6*b*). Eosinophils also expressed MHC class II molecules (Fig. 6*c*) and were positive for peptide-loaded MHC class II molecules (Fig. 6*d*). These results confirmed recent data that show eosinophils from the lungs of sensitized Ag-challenged mice express MHC class II and the two B7 proteins intimately involved in eliciting costimulatory signals during T cell priming with Ag (41, 42). We (unpublished observation) and others (9) have also shown that Ag-loaded eosinophils can induce the proliferation of T cells in culture. Thus, knowing that eosinophils can express the molecules associated with Ag presentation, stimulate T cell proliferation, and localize to T cell-rich regions of LALN during allergen provocation of the lung, we next tested whether eosinophils could present Ag to in vitro-polarized CD4$^+$ Th2 cells. Purified eosinophils from IL-5-transgenic mice were able to induce IL-4, IL-5, and IL-13 production in Th2 cells in an Ag-specific manner (Fig. 7, *a–c*). These cells did not produce IFN-γ on stimulation (results not shown). Thus, eosinophils are able to induce proinflammatory cytokine secretion from CD4$^+$ Th2 cells polarized in vitro.

Next, we determined whether eosinophils and T cells from allergic mice could also interact to induce IL-5 secretion. Eosinophils were purified from the BALF of the allergic lung and incubated with CD4$^+$ T cells that were isolated from the spleens of the same allergic mice. Incubation of either CD4$^+$ T cells or eosinophils alone in medium resulted in no appreciable production of

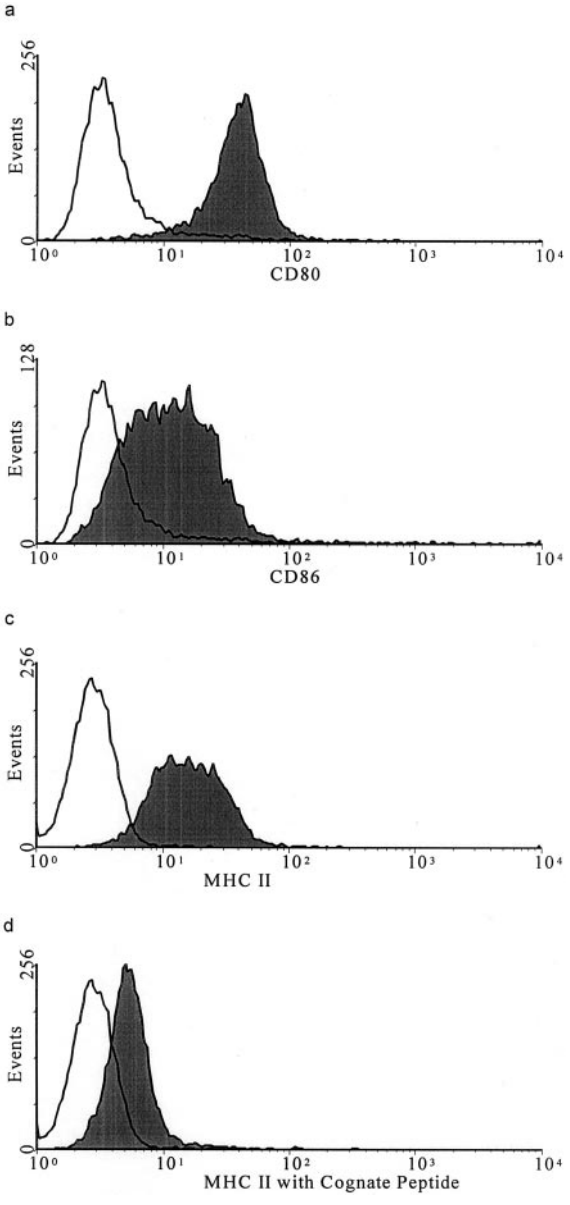

FIGURE 6. Mouse eosinophils express class II MHC and T cell costimulatory molecules. BALF eosinophils from allergic mice are CD80 (*a*, filled histogram) and CD86 (*b*, filled histogram) positive. Ninety percent of BALF eosinophils are class II MHC positive (*c*, filled histogram) and 50% are positive for class II MHC with cognate peptide (*d*, filled histogram). Unlabeled cells are represented by the open histogram.

IL-5 (Fig. 7*d*). Addition of eosinophils (Eos) and exogenous OVA (OVA) to $CD4^+$ T cell cultures resulted in IL-5 production (Eos/CD4/OVA) (Fig. 7*d*), the magnitude of which increased as more eosinophils were added. Notably, the levels of IL-5 produced in Eos/CD4/OVA cultures were comparable to those induced in cultures containing similar numbers of splenocytes (CD4/APC/OVA) (Fig. 7*d*). Our data show that eosinophils from the allergic lung can stimulate and enhance IL-5 secretion from $CD4^+$ T cells in an Ag-dependent (OVA) manner.

Adoptive transfer of eosinophils loaded exogenously with Ag to naive mice induces Th2 cell-driven allergic disease of the lung

We have shown that eosinophils are spatially localized to pulmonary compartments which encounter Ag in the allergic lung. We have also shown that these eosinophils appear to function as APCs and activating Ag-specific $CD4^+$ Th2 cells and this suggests that eosinophils have the potential to amplify allergic inflammation. We also speculated that eosinophils might contribute to priming of Th2 cells. Therefore, we transferred eosinophils that were pulsed with OVA in vitro (Ag-loaded (Ag^+ eos)) or were untreated (Ag^- eos) to the peritoneal cavities of naive mice 15 days before exposure to an OVA aerosol (Fig. 8). Ag inhalation resulted in the induction of blood eosinophilia and the concomitant recruitment of eosinophils to the airways lumen in recipients that received Ag^+ eos but not Ag^- eos (Fig. 8, *a* and *b*). Characterization of Ag-specific (OVA) T cell responses from LALN cells showed IL-5 and IL-13 secretion from Ag^+ eos recipients only (Fig. 8, *c* and *d*). Normal priming for Th2 responses by delivery of free soluble Ag i.p. results in a robust Ab titer over this time frame in response to subsequent Ag inhalation (36). This was not evident in these transfer experiments. ELISA-based analysis of sera from both groups of mice revealed no OVA-specific IgG1 was produced (data not shown), suggesting that passive transfer of OVA to professional endogenous APCs, from transferred eosinophils, was not the basis for the induction of disease. Histological sections of lung from Ag^+ eos mice showed the characteristic features of allergic disease that are induced by OVA sensitization and airway challenge (36). Notably, lung sections from Ag^+ eos but not Ag^- eos recipients were characterized by a pronounced eosinophil infiltrate in and around the airways, mucus plugging, and cellular disruption of the airways epithelium (Fig. 8, *e* and *f*).

Discussion

Eosinophils are prominent features in allergic mucosal surfaces of the lung and gastrointestinal tract (1, 2). Currently, these cells are primarily thought to act as effector cells, inducing pathological changes through the release of granular proteins and proinflammatory mediators. However, under normal physiological conditions, tissue eosinophils do not induce disease and their numbers and activation status are tightly regulated. The presence of eosinophils in mucosal surfaces at baseline, in association with their ability to release cytokines and express molecules that engage T cells, suggests that these cells may also have immunomodulatory functions. Indeed, limited in vivo investigations suggest that eosinophils and mast cells have the potential to direct immune responses associated with allergic inflammation and parasitic infestation. In this investigation, we show that eosinophils traffic to sites of Ag deposition (airways lumen) and T cell expansion (LALN) in the allergic lung in response to Ag provocation of the airways. These lumenal eosinophils have the ability to sequester and process Ag and to express class II MHC and the T cell costimulatory molecules CD80 and CD86. Ag-loaded eosinophils also have the ability to induce cytokine production from $CD4^+$ Th2 cells in an Ag-dependent manner. We further demonstrate the capacity of eosinophils to act as immunomodulatory cells by showing that allergic disease of the lung can be induced in naive mice by the transfer of eosinophils loaded with Ag. Collectively, our data suggest that eosinophils play an important role in propagating allergic disease by regulating $CD4^+$ T cell function within the pulmonary microenvironment.

To investigate the potential role of eosinophils as immunomodulatory cells of allergic disease, we initially characterized their distribution in pulmonary compartments after the induction of allergic inflammation. Ag challenge of the respiratory mucosal surface resulted in the localization of eosinophils at sites of Ag deposition (airways lumen) as well as specialized regions involved in Ag presentation to T cells (regional lymph nodes). The recruitment of eosinophils to these compartments was rapid and correlated with the induction of a systemic eosinophilia. Eosinophil recruitment to

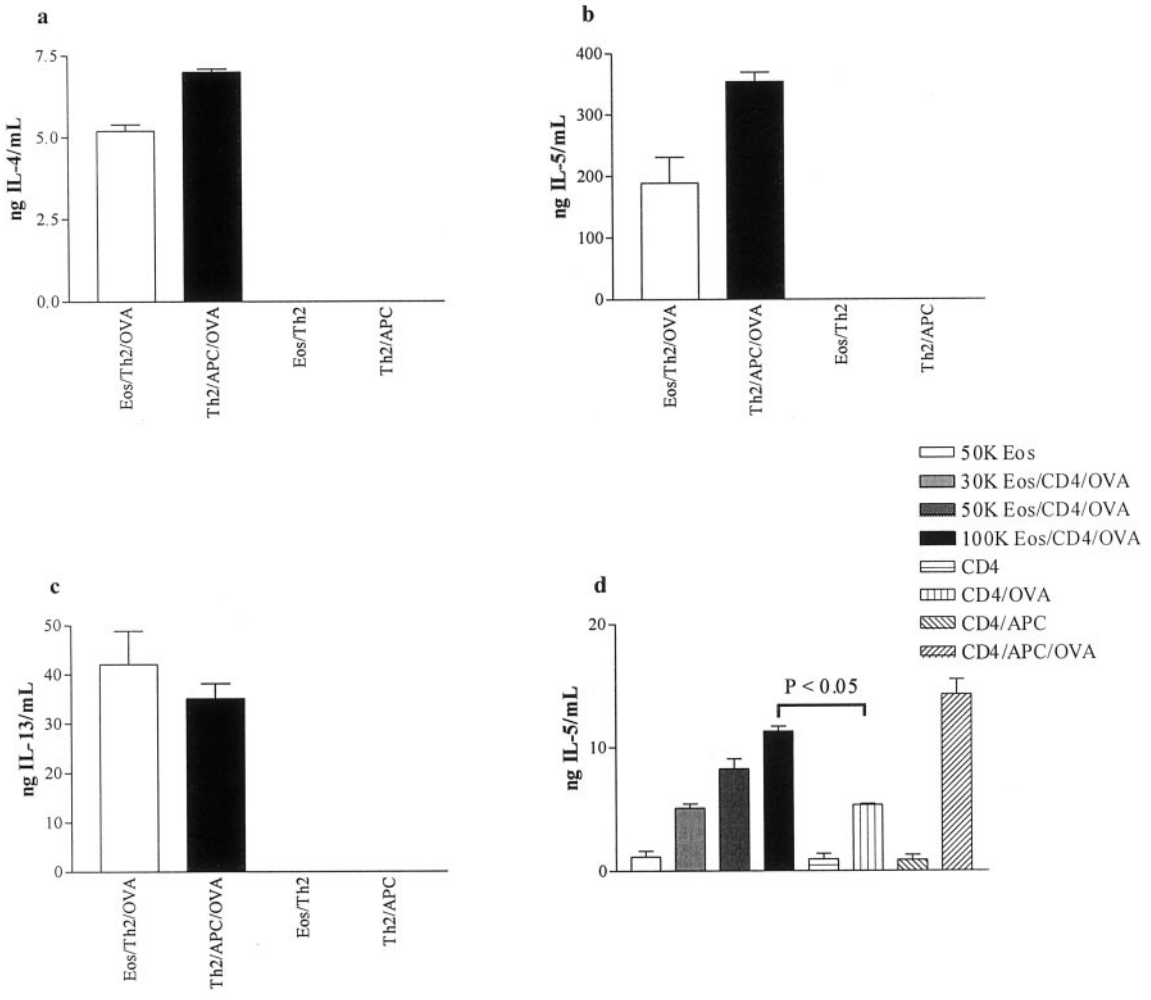

FIGURE 7. Eosinophils stimulate cytokine production from Th2 cells generated in vitro and $CD4^+$ T cells from allergic mice. Coculture of eosinophils (5×10^5 cells/well) isolated from IL-5-transgenic mice with in vitro-polarized Th2 cells (5×10^5 cells/well) resulted in elevated levels of IL-4 (*a*), IL-5 (*b*), and IL-13 (*c*). Th2 cells incubated with eosinophils (Eos/Th2/OVA) or mitomycin C-treated splenocytes (Th2/APC/OVA) in the presence of OVA produced similar levels of cytokines. Th2 cells incubated with eosinophils (Eos/Th2) or mitomycin C-treated splenocytes (Th2/APC) without OVA did not promote measurable production of cytokine. Eosinophils were also isolated from the allergic lung and cultured in various numbers with $CD4^+$ T cells isolated from the spleens of these allergic mice (*d*). $CD4^+$ T cells secreted IL-5 when stimulated with APCs ($CD4^-$ fraction) (CD4/APC/OVA) and purified BALF eosinophils (EOS/CD4/OVA) from allergic mice in the presence of exogenous OVA. Increasing numbers of eosinophils from 30,000 (30K) to 100,000 (100K) resulted in greater production of IL-5 by $CD4^+$ T cells.

lymph nodes after Ag loading is not a recent observation, with the earliest evidence we could find dating back to 1966 (43). Notably, significant numbers of eosinophils were present in all three compartments (blood, airways lumen, and LALN) before Ag inhalation (Fig. 1), supporting a role for this leukocyte in immunological responses associated with immune surveillance and, potentially, T cell expansion.

Although it is difficult to determine from our data the route of eosinophil migration within the lung compartment, it was observed that lumenal eosinophil numbers initially decreased while lymph node numbers increased in the initial phases (over the first 9 h) after Ag deposition in the lung (Fig. 1). The ability of eosinophils to migrate from the airways lumen to local lymph nodes had been recently demonstrated (9). The installation of fluorescently labeled eosinophils, taken from the allergic lung or from the peritoneal cavity of IL-5-transgenic mice, into the tracheal lumina migrated into regional lymph nodes (9). Indeed, the ability of eosinophils to move bidirectionally across the epithelium has been demonstrated in vitro with intestinal monolayers. We extend these investigations by demonstrating that lumenal eosinophils not only migrate into pulmonary tissues but also sequester and process Ag that is deposited within the airways. Lumenal eosinophils also expressed class II MHC and were loaded with cognate peptide. Eosinophils isolated from the allergic lung also expressed CD80 and CD86. Both of these molecules have been implicated, to different degrees, in the mechanism for the induction of allergic inflammation (41, 42, 44–47). Furthermore, eosinophils have previously been shown to activate Ag-specific T cells in coculture in a CD80- and CD86-dependent manner (13). Collectively, these experiments demonstrate that eosinophils have all of the required molecular machinery to directly engage and activate T cells.

These data suggest that eosinophils residing within the airways mucosa and lumen have the ability to sample the local microenvironment before translocating to regional lymph nodes to engage in immunomodulatory responses. The mechanism regulating the trafficking of eosinophils from the airways lumen to regional pulmonary lymph nodes has not yet been elucidated, but appears to be independent of the CCR3 receptor, suggesting that other chemokine or adhesion pathways modulate this process (9). Eosinophils may change their adhesion molecule profile after transmigration

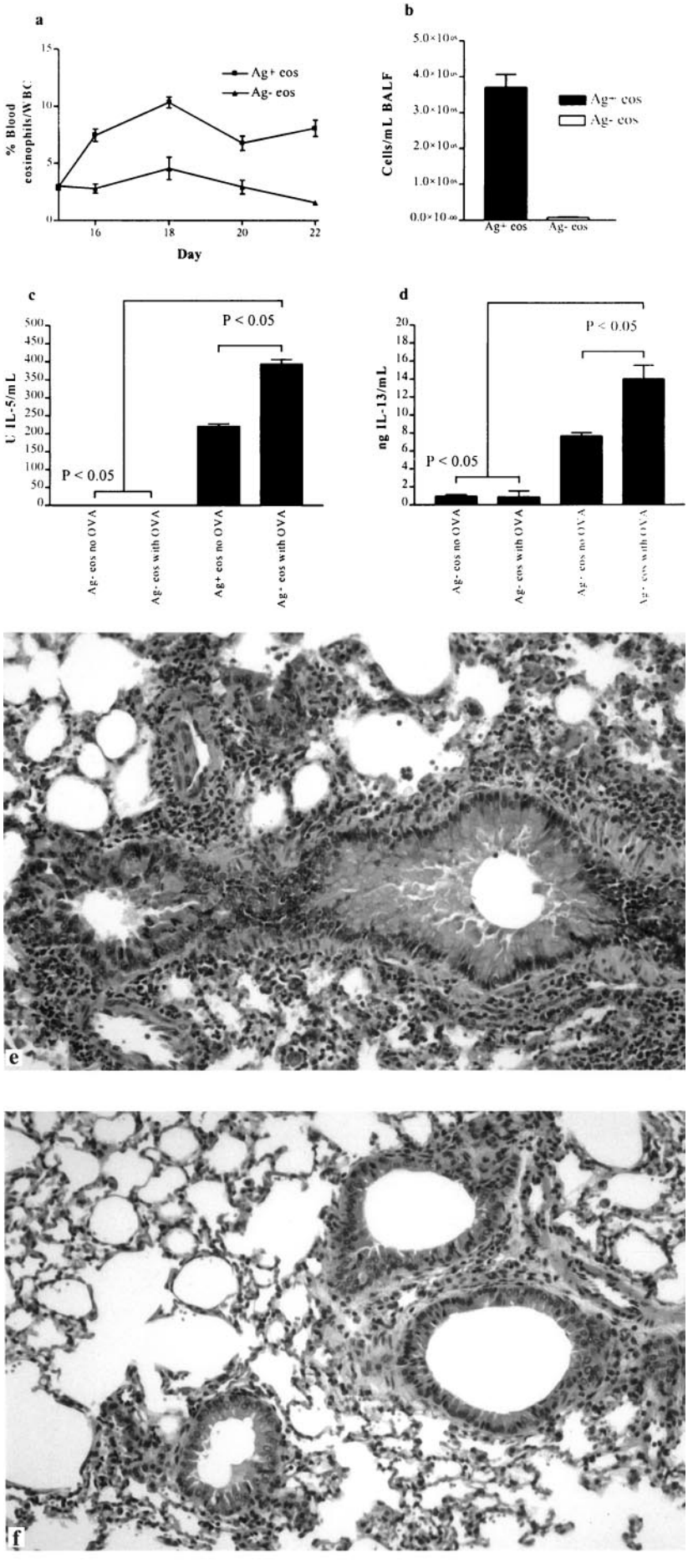

FIGURE 8. Ag-loaded eosinophils induce allergic disease in naive mice. Recipients of Ag-loaded eosinophils (Ag^+ eos) developed a pronounced blood (*a*) and BALF (*b*) eosinophilia in response to Ag provocation. Transfer of Ag^+ eos also generated a memory response with a Th2 cytokine profile in naive mice. Stimulation with OVA of LALN cells from Ag^+ eos recipients (Ag^+ eos with OVA) shows Ag-specific IL-5 (*c*) and IL-13 (*d*) secretion compared with controls (Ag^+ eos no OVA). Stimulated LALN cells from mice injected with Ag-free eosinophils (Ag^- eos no OVA and Ag^- eos with OVA) did not secrete IL-5 or IL-13 in response to OVA. Marked cellular infiltrates and changes in lung morphology (*e*) were observed in recipients of eosinophils (Ag^+) following Ag provocation in comparison to control (Ag^-) mice (*f*).

across the epithelial surface, which may facilitate the migration of these cells back into pulmonary compartments. Differential expression of adhesion and accessory molecules by eosinophils has been observed between blood- and tissue-derived cells, suggesting that these cells change phenotype as they migrate across the vascular bed (4–6, 8).

Investigations in vitro indicate that human eosinophils also process the molecular machinery required to communicate with T cells (7, 28, 41, 42, 44–48). To examine the ability of eosinophils to promote Th2 immunity, we cocultured eosinophils with Ag-specific $CD4^+$ Th2 cells that had been polarized in vitro and found appreciable amounts of IL-4, IL-5, and IL-13 in the incubation medium after stimulation with cognate Ag. These cytokines play key roles in the pathogenesis of allergic responses and in host defense, and are primarily thought to be secreted from Th2 cells (25). Eosinophils and $CD4^+$ T cells derived from allergic mice also directly communicate in an Ag-specific manner to induce IL-5 production. Under these culture conditions, eosinophils also induced the proliferation of the purified $CD4^+$ T cells (results not shown) (9). Thus, bidirectional signaling events may occur between eosinophils and T cells, resulting in the release of IL-4, IL-5, and IL-13, which may amplify inflammatory cascades within allergic tissue. However, eosinophils also express a range of proinflammatory cytokines, including those of the Th2-like profile (14, 17–19, 23, 49, 50). Thus, we cannot rule out the possibility that these cytokines may also be derived from eosinophils as well as Ag-reactive T cells.

The transfer of Ag-loaded eosinophils to naive mice also predisposed to the induction of allergic disease of the lung after subsequent challenge with the Ag delivered as an aerosol. Allergic disease in recipient mice resembled that observed in sensitization and allergen challenge models and was characterized by an intense eosinophil infiltrate in the airways, mucus hypersecretion (data not shown), and tissue perturbation. Eosinophil transfer also primed the $CD4^+$ T cell compartment to the Th2 phenotype. Although allergic disease of the lung was induced in recipient mice, no Ag-specific Abs were produced in the time frame of experimentation, suggesting that priming for allergic disease occurred by interactions between eosinophils and T cells and not by Ag leakage and transfer to professional APCs. These investigations suggest that eosinophils have the potential to not only activate Th2 cells to release disease-modulating cytokines but also to participate in priming the immune system for allergic responses.

Although limited investigations suggest that eosinophils can potentially direct immune responses through the release of cytokines and by acting as APCs, they are primarily thought of as effector cells for the elimination of parasites and in inducing allergic disease. Our data support the concept that eosinophils act as immune effectors but also demonstrates that these cells modulate Th2 immunity by acting as APCs. It has been suggested that eosinophils have distinct spatial locations in tissues and functional characteristics that endow them with unique roles as APCs in contrast to APCs such as dendritic cells, macrophages, and B cells (9). Their location at mucosal surfaces (respiratory, gastrointestinal, lower genitourinary tracts (51) and in the airways lumen of the allergic lung) positions them to rapidly encounter foreign Ags and inhaled allergens. Eosinophils are phagocytic and rapidly encounter particulate Ags at sites of deposition (52). Indeed, in the respiratory tract, inhaled allergens are primarily particulate (53), and unlike B cells and dendritic cells, eosinophils readily interact with this form of Ag (54). Although particulate aeroallergens are processed by macrophages, alveolar macrophages are not effective APCs (55–57). The ability of eosinophils to deal with particulate Ags may stem from their immune role in host defense against large multicellular targets. Shi et al. (9) also suggest that the colocalization of allergen-specific IgE, IgG, IgA, and eosinophils (which express receptors for these Igs) in mucosal surfaces may facilitate enhancement of Ag uptake and presentation as is observed for other APCs (58, 59).

Thus, eosinophils appear to have a number of characteristics that endow them with the ability to processes inhaled particulate Ags/allergens that would provide a basis for the immune system to utilize this cell as a functional APC. It is tempting to speculate that eosinophils within the lumen and mucosal surface of the asthmatic lung can sequester and process inhaled allergens and subsequently engage allergen-specific Th2 cells within local lymph nodes and the airways wall to induce and/or exacerbate the allergic response.

In conclusion, we have shown that eosinophils traffic to sites of Ag deposition and T cell education in the allergic lung. These eosinophils express class II MHC and T cell costimulatory molecules and have the ability to sequester, process, and present airway-derived Ag. In addition, Ag-specific Th2 cells may directly engage eosinophils pulsed with cognate Ag to promote the production of cytokines (IL-4, IL-5, and IL-13) that are intimately involved in allergic responses and host defense. Of importance is the transfer of Ag-loaded eosinophils to naive mice primed for induction of allergic disease of the lung in response to Ag inhalation. Thus, eosinophils have the potential to not only activate Th2 cells to release disease-modulating cytokines but also to participate in priming the immune system for allergic responses. These investigations highlight the potential of eosinophils to not only act as terminal effector cells but also to actively amplify allergic responses by promoting Th2 cell immunity.

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