Ablation of eosinophils leads to a reduction of allergen-induced pulmonary pathology

J. PAUL JUSTICE,1 MICHAEL T. BORCHERS,2 JEFFREY R. CROSBY,1 EDITH M. HINES,1 HUAHAO H. SHEN,1,3 SERGEI I. OCHKUR,2 MICHAEL P. MCCGARRY,2 NANCY A. LEE,1 AND JAMES J. LEE2

1Division of Hematology/Oncology and 2Pulmonary Medicine, Department of Biochemistry and Molecular Biology, S. C. Johnson Medical Research Building, Mayo Clinic Scottsdale, Scottsdale, Arizona 85259; and 3Department of Respiratory Medicine, Second Hospital, Zhejiang University College of Medicine, HangZhou 310009, People’s Republic of China

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Ablation of eosinophils leads to a reduction of allergen-induced pulmonary pathology. Am J Physiol Lung Cell Mol Physiol 284: L169–L178, 2003. First published September 13, 2002; 10.1152/ajplung.00260.2002.—A strategy to deplete eosinophils from the lungs of ovalbumin (OVA)-sensitized/challenged mice was developed using antibody-mediated depletion. Concurrent administration [viz. the peritoneal cavity (systemic) and as an aerosol to the lung (local)] of a rat anti-mouse CCR3 monoclonal antibody resulted in the abolition of eosinophils from the lung such that the airway lumen was essentially devoid of eosinophils. Moreover, perivascular/peribronchial eosinophil numbers were reduced to levels indistinguishable from saline-challenged animals. This antibody-mediated depletion was not accompanied by effects on any other leukocyte population, including, but not limited to, T cells and mast cells/basophils. In addition, no effects were observed on other underlying allergic inflammatory responses in OVA-treated mice, including OVA-specific immunoglobulin production as well as T cell-dependent elaboration of Th2 cytokines. The ablation of virtually all pulmonary eosinophils in OVA-treated mice, including OVA-specific immunoglobulin production as well as T cell-dependent elaboration of Th2 cytokines. The ablation of virtually all pulmonary eosinophils in OVA-treated mice (i.e., without concurrent effects on T cell activities) resulted in a significant decrease in mucus accumulation and abolished allergen-induced pulmonary pathologies arise from studies manipulating in vivo IL-5 levels, including IL-5 knockout mice (18), antibody neutralization studies (see for example Ref. 30), and IL-5 overexpression in transgenic mice (32). These studies each relied on the premise that IL-5 activities in the mouse are limited to proliferative and survival effects on eosinophils, activities that elicit B cell maturation, and potential agonist effects directly on airway smooth muscle (22, 34). However, because ovalbumin (OVA)-induced pulmonary pathologies are not diminished in B cell-deficient mice (29), the loss of pulmonary pathologies in IL-5-deficient animals has been assumed to be a consequence of IL-5-mediated effects on eosinophils alone. For example, studies using IL-5 knockout mice (18) or neutralization studies using the TRFK-5 antibody (23) each demonstrated that the loss of IL-5-mediated pulmonary eosinophilia was accompanied by the elimination of AHR. Moreover, a study investigating allergen provocation in the mouse also demonstrated that IL-5 was
necessary for the late phase of allergen-mediated bronchoconstriction (6). Interestingly, the effects of abolishing IL-5 were not necessarily limited to pulmonary pathophysiology as Kung and colleagues (30) demonstrated that treatment of mice with TRFK-5 antibodies significantly reduced goblet cell metaplasia/mucus production.

In an attempt to avoid the pleiotropic effects of neutralizing IL-5, eosinophils were uniquely ablated in the lungs of allergen-sensitized/challenged mice through a strategy using a depleting rat monoclonal antibody specific for mouse CCR3. CCR3 is a chemokine receptor whose principle ligands (i.e., eotaxin-1 and -2) are potent chemoattractants displaying a unique specificity for eosinophils (44). In the mouse, CCR3 expression appears to be restricted to eosinophils. Moreover, systemic administration of a depleting anti-CCR3 monoclonal antibody was capable of inducing a targeted reduction in peripheral blood, although potential effects on all leukocytes were not examined (20). In contrast to the apparent eosinophil specificity of CCR3 antibodies, the loss of CCR3 function in knockout mice led to a 4- to 12-fold increase in lung tissue mast cells/basophils following OVA sensitization/aerosol challenge, suggesting to the authors that CCR3 expression may also occur on these granulocytes (27). The specificity and utility of depleting eosinophils using an anti-CCR3 antibody strategy were determined in this study by modifying the previously published protocol using this reagent to yield a methodology that abolishes eosinophils from the airway lumen of OVA-sensitized/challenged mice and reduces the perivascular/peribronchial eosinophilia to levels indistinguishable from naive saline-challenged animals. Significantly, the data show that no other cell types, including mast cells/basophils, are affected as a consequence of antibody treatment. In particular, lymphocyte activities responsible for the underlying inflammatory responses associated with allergen challenge were intact, including allergen-specific immunoglobulin production (B cell functions), T cell-mediated elaboration of pulmonary Th2 cytokines, and ex vivo allergen-dependent memory responses of isolated T cells. Despite leaving T cell functions apparently intact, the selective ablation of eosinophils attenuates allergen-induced goblet cell metaplasia/mucus production and eliminates AHR in response to cholinergic receptor agonist. These data suggest a causative relationship exists between pulmonary eosinophils and allergen-induced pulmonary pathologies and potentially highlight a synergy between CD4+ T cell activities and eosinophil effector functions (reviewed in Ref. 33), both of which are apparently required for the development of the pathologies associated with allergic respiratory inflammation.

**MATERIALS AND METHODS**

**Animals.** C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Experimental protocols were performed on mice 8–12 wk old maintained in ventilated microisoler cages housed in a specific pathogen-free animal facility. Procedures and studies involving animals were conducted in accordance with National Institutes of Health and Mayo institutional guidelines.

**OVA sensitization and challenge.** Mice were sensitized and challenged with chicken OVA as previously described (12). Briefly, on days 0 and 14, all animals were injected (100 μl ip) with OVA (20 μg crude grade IV; Sigma, St. Louis, MO) emulsified in 2.25 mg of aluminum hydroxide/magnesium hydroxide (Pierce, Rockford, IL). On days 24–26, animals were exposed for 20 min to an OVA aerosol (1% wt/vol OVA in saline) generated with an ultrasonic nebulizer (DeVilbiss, Somerset, PA). Control animals received a saline-only aerosol. The mice were assessed for pulmonary cellular infiltrates, histopathologies, and lung function on day 28.

**Administration of anti-mouse CCR3 monoclonal antibodies.** On days 24–26 of the OVA protocol, animals were administered rat anti-mouse CCR3 antibodies by two independent routes. Before each aerosol OVA challenge, mice were injected (intraperitoneally) with 150 μg of rat anti-mouse CCR3 monoclonal antibodies [6S2-19-4; IgG2b, a kind gift of Dr. R. Coffman, DNAX, Palo Alto, CA)]. In addition, 50 μg of anti-mouse CCR3 antibodies were mixed with the OVA solution generating the nebulant (final concentration: 1 μg/ml) and administered with each of the three OVA challenges. Control mice were exposed to equivalent amounts of nonspecific rat IgG2b (Pharmingen, Torrence, CA) by both routes of administration.

**Collection of bronchoalveolar lavage fluid and serum, isolation of splenocytes and marrow-derived cells, and the enumeration of leukocytes.** On day 28 of the OVA protocol, animals were killed, the tracheas were cannulated, and the lungs were lavaged (3 × 0.5 ml) with ice-cold PBS/2% fetal calf serum. The methods and assessments of bronchoalveolar lavage (BAL) cells have been described previously (32). Cell-free BAL fluid samples were stored at −80°C before assessment of cytokine levels by ELISA.

Splenocytes and marrow-derived cells were recovered and quantified, including cell differentials, as previously described (35). In some experiments, specific leukocyte populations were identified and expressed as the percent of total cells analyzed (1 × 10⁶) by flow cytometry. CD3+, CD4+, and CD8+ cells (T lymphocytes and specific subpopulations), B220+ (CD45R) (B lymphocytes), MAC-1+ (macrophage/monocytes), F4/80+ (macrophage/monocytes, eosinophils), and NK1.1+ (NK cells) were identified/quantified by staining with fluochrome-conjugated antibodies (Pharmingen, San Diego, CA). Flow cytometry was performed on a FACSscan using CellQuest data-acquisition/analysis software (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood was recovered by cardiac puncture and allowed to clot at room temperature for 1 h. Cell counts and differentials were performed following centrifugation at 4°C (35), assessing a minimum of 300 cells. Serum samples were stored at −80°C before determination of immunoglobulin levels by ELISA.

**Allergen-mediated histological changes in the lung and immunohistochemical detection of eosinophils in paraffin-embedded tissue.** Pulmonary histology, including mucus content of the airway epithelium, was assessed from tissues excised and fixed in 10% formalin [lungs were inflated with a fixed volume (0.5 ml) of fixative]. The lung samples (day 28) were washed free of formalin and subsequently dehydrated through an ethanol series before equilibration in xylene and embedding in paraffin. Sections (4 μm) were stained with 0.1% toluidine blue to identify and localize pulmonary mast cells/basophils. In addition, lung sections were also stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin/methylgreen to assess goblet cell metaplasia/mucus production. Parasagittal sections were analyzed by

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bright-field microscopy, and analysis of the mucus content of the airway epithelium of mice from different groups was based on the evaluation of airways (both proximal and distal)/mouse (n = 8 animals/group). Relative comparisons of mucus content between cohorts of animals were performed using the imaging program Image ProPlus (Media Cybernetics, Silver Spring, MD). The data were quantified as an airway mucus index: [(average PAS staining intensity of the airway epithelium) × (area of airway epithelium staining with PAS)/ (total area of the conducting airway epithelium)] × (total number of airways assessed).

Infiltrating lung eosinophils were assessed by immunohistochemistry using a rabbit polyclonal anti-mouse eosinophil major basic protein antisera (13, 32). Immunocytochemical staining was performed with DAB-peroxidase detection reagents (Vector Laboratories, Burlingame, CA) as described previously (13).

Detection of serum IgG1 and IgE. Serum concentrations of total IgE were determined using an immunoassay kit (Endogen, Cambridge, MA) according to the manufacturer’s instructions. In addition, OVA-specific IgG1 levels were determined as previously described (39). The limits of detection associated with each assay are 2 ng/ml and 0.2 A410 OD U/ml, respectively.

Cytokine assays. Cytokine levels in BAL fluid were determined by ELISA. Mouse IL-4, IL-5, and IL-13 levels were assessed using immunoassay kits (Endogen) as per the manufacturer’s instructions. The limits of detection for each assay were: IL-4 = 10 pg/ml, IL-5 = 10 pg/ml, and IL-13 = 30 pg/ml.

Ex vivo cytokine production by allergen-stimulated splenocytes. Mice were sensitized and either aerosol challenged with OVA or challenged with OVA concurrent with the administration of rat anti-mouse (rm) CCR3 mAb as described above. On day 28 (2 days following the last aerosol challenge), spleens were removed and cells were suspended in RPMI-1640 media containing 5% FCS, penicillin (100 U/ml), and streptomycin (100 U/ml) and cultured in 96-well microtiter plates at a concentration of 5 × 10^6 cells/ml in the presence of 20 μg/ml of OVA. The capacity of lymphocytes in the spleen suspensions to produce both Th1 (e.g., IFN-γ) and Th2 (e.g., IL-4 and IL-5) cytokines in response to OVA stimulation was assessed using immunoassay kits (Endogen) as per the manufacturer’s instructions. The data were quantified as an aerosol to the lung (local) in conjunction with systemic administration to the peritoneal cavity. Systemic/local delivery of rmCCR3 mAb, similar to systemic administration alone, resulted in an ~80% reduction of bone marrow eosinophils (relative to nonspecific IgG-treated mice) 48 h following the last OVA aerosol challenge [1.75 ± 0.29 (% of marrow) vs. 10.25 ± 1.44, respectively]. However, this dual administration of rmCCR3 mAb eliminated spleen and peripheral blood eosinophils without any significant effects on the numbers of lymphocytes, monocytes/macrophages, or neutrophils in these peripheral compartments (Fig. 2). Enumeration of the BAL cellularity following OVA challenge showed that this depletion of eosinophils also occurred in the airway lumen, again without any effects on other leukocytes in the airways (Fig. 3). It is noteworthy that the loss of eosinophils in six or eight rmCCR3 mAb-treated mice was absolute as these cells were absent even from comprehensive

RESULTS

Rat anti-mouse CCR3 monoclonal antibodies selectively ablate eosinophils. A preliminary assessment of the cell-depleting specificity of an rmCCR3 mAb in vivo was performed using a protocol similar to the antibody-dependent complement-mediated cell lysis-depleting strategy of Grimaldi and colleagues (20). RmCCR3 mAb (150 μg) was administered (intraperitoneally) to naive mice on 3 successive days; control animals received nonspecific rat IgG2b. These data showed that 24 h following the final antibody administration eosinophils were reduced in the peripheral blood by >90% (Fig. 1). In contrast to the significant decrease of blood eosinophils, administration of rmCCR3 mAb had no effect on the numbers of the remaining leukocytes (i.e., lymphocytes, monocytes, and neutrophils), demonstrating the specificity of rmCCR3 mAb treatment for only eosinophils.

A strategy of systemic and local administration of rmCCR3 mAb uniquely abolishes allergen-induced airway eosinophil recruitment. Although circulating eosinophils were effectively eliminated in OVA-sensitized mice systemically administered (intraperitoneally) rmCCR3 mAb concurrently with the OVA aerosol challenges, this methodology only decreased, i.e., did not abolish, eosinophil numbers in the lung (data not shown). Investigations of alternative administration strategies, in contrast, showed that it was possible to completely block the allergen-induced recruitment of eosinophils to the lungs of mice through a dual-administration strategy in which rmCCR3 mAb was delivered as an aerosol to the lung (local) in conjunction with systemic administration to the peritoneal cavity. Systemic/local delivery of rmCCR3 mAb, similar to systemic administration alone, resulted in an ~80% reduction of bone marrow eosinophils (relative to nonspecific IgG-treated mice) 48 h following the last OVA aerosol challenge [1.75 ± 0.29 (% of marrow) vs. 10.25 ± 1.44, respectively]. However, this dual administration of rmCCR3 mAb eliminated spleen and peripheral blood eosinophils without any significant effects on the numbers of lymphocytes, monocytes/macrophages, or neutrophils in these peripheral compartments (Fig. 2). Enumeration of the BAL cellularity following OVA challenge showed that this depletion of eosinophils also occurred in the airway lumen, again without any effects on other leukocytes in the airways (Fig. 3). It is noteworthy that the loss of eosinophils in six or eight rmCCR3 mAb-treated mice was absolute as these cells were absent even from comprehensive

Fig. 1. Intraperitoneal administration of rmCCR3 mAb (6S2-14-9) into naive mice leads to a significant reduction in peripheral eosinophil levels without effects on the numbers of other leukocytes. Peripheral blood leukocytes were assessed following 3 consecutive days of receiving intraperitoneal injections of either nonspecific rat IgG2b (ns-IgG) or rmCCR3 mAb (α-CCR3). Data represent means ± SE (n = 5 animals/group). Mono, monocyte; Lym, lymphocyte; Eos, eosinophil; Neu, neutrophil; WBC, white blood cell. *P < 0.05.
surveys of entire cytospin preparations. Treatment of OVA-sensitized/challenged mice with rMCCR3 mAb also eliminated the allergen-induced increase of tissue eosinophils in the perivascular and peribronchial regions as well as the airway submucosa (Fig. 4). Complete surveys of several (i.e., 2–4) parasagittal lung sections from rMCCR3 mAb-treated mice (n = 5) revealed that eosinophil levels in these mice were reduced to levels indistinguishable from naive saline-challenged animals (Fig. 5). Significantly, the treatment of OVA-sensitized/challenged mice with rMCCR3 mAb did not lead to a loss of lung tissue mast cells/basophils (Fig. 6), demonstrating a lack of significant expression of CCR3 on these granulocytes. Moreover, the ablation of eosinophils following treatment with rMCCR3 mAb was also not accompanied by changes in the number of pulmonary mast cells/basophils relative to OVA-treated mice administered nonspecific control antibody (data not shown).

Ablation of circulating and pulmonary eosinophils does not affect lymphocyte-mediated acquired immune responses to OVA, including immunoglobulin and Th2 cytokine production. The potential expression of CCR3 on other leukocytes, particularly lymphocytes (1), held open the possibility that the loss of allergen-induced pulmonary eosinophilia in mice treated with anti-CCR3 antibodies resulted from effects on underlying immune responses and not through the direct targeting of eosinophils. However, two measures of lymphocyte function demonstrated that administration of rMCCR3 mAb had no discernable effect(s) on either allergen-induced B cell functions or T cell activities. Assessments of serum immunoglobulin production (i.e., B cell/CD4$^+$ T cell functions) showed that administration of rMCCR3 mAb concurrent with OVA aerosol challenge did not affect either the significant rise in total IgE occurring in allergen-treated mice (Fig. 7A) or the appearance of OVA-specific IgG1 (Fig. 7B). Allergen-mediated pulmonary production of Th2 cytokines (i.e., CD4$^+$ T cell activities) was also unaffected by administration of rMCCR3 mAb. The BAL levels of IL-4, IL-5, and IL-13 in rMCCR3 mAb-treated mice following the last OVA challenge were indistinguishable from control animals receiving nonspecific IgG2b (Fig. 8A). In addition, Th1 cytokines such as IFN-$\gamma$ remained at low/undetectable levels in the BAL of all the groups examined (data not shown). Moreover, ex vivo restimulation of splenocytes with OVA (i.e., memory responses) was also unaffected as equivalent levels of Th2 cytokines (i.e., IL-4 and IL-5), as well as IFN-$\gamma$, were observed from splenocytes of wild-type and rMCCR3 mAb-treated mice (Fig. 8B).

The selective loss of airway eosinophils significantly attenuates pulmonary histopathologies, including OVA-

![Figure 2](http://ajplung.physiology.org/)

**Fig. 2.** Concurrent administration of aCCR3 viz. the peritoneal cavity (systemic) and as an aerosol delivered to the lung (local) led to the ablation of eosinophils from the periphery. The leukocyte composition, excluding erythroblasts, of spleen (A) and of peripheral blood (B) was assessed in groups of mice representing saline- and ovalbumin (OVA)-sensitized/aerosol-challenged animals, as well as OVA-sensitized/aerosol-challenged mice administered either ns-IgG or aCCR3 concurrent with the OVA aerosol challenges. Data demonstrate that, unlike saline- and OVA-challenged mice or OVA-treated mice administered ns-IgG, dual administration of aCCR3 eliminated eosinophils from both the spleen and peripheral blood. Data represent means ± SE (n = 5–8 animals/group). No statistically significant differences were observed in any noneosinophil leukocyte population between the control groups of mice and animals administered aCCR3. ND, not detectable; Mono/Mφ, monocyte/macrophage.

![Figure 3](http://ajplung.physiology.org/)

**Fig. 3.** OVA-induced eosinophilia of the airway lumen was abolished as a consequence of dual administration (i.e., intraperitoneal injection/aerosol inhalation) of aCCR3. The cellularity of bronchoalveolar lavage (BAL) fluid was assessed (n = 5–10 animals/group) in saline- and OVA-sensitized/aerosol-challenged animals, as well as OVA-sensitized/aerosol-challenged mice administered either ns-IgG or aCCR3 concurrent with the OVA aerosol challenges. The cellularity of each animal cohort is expressed as the product of the total number of cells recovered and the percentages of each cell type derived from differentials of Wright-stained cytocentrifuge preparations. Data represent means ± SE, counting a minimum of 300 cells. *P < 0.05.
induced goblet cell metaplasia/mucus production. 

RmCCR3 mAb-treated mice displayed a generalized reduction in airway inflammation typified by a decrease in airway epithelial hypertrophy (see for example Fig. 4, right). In addition, mucous levels in the airways of RmCCR3 mAb-treated mice displayed a 75% decrease relative to OVA-treated animals receiving nonspecific rat IgG_{2b} (Fig. 9). However, despite the inhibition of goblet cell metaplasia/mucus production in RmCCR3 mAb-treated mice, in absolute terms the observed metaplasia/mucus production was nonetheless elevated relative to saline-challenged control mice, demonstrating that eosinophil-dependent and -independent mechanisms are necessary for the levels of goblet cell metaplasia/mucus production observed in OVA-treated wild-type mice.

AHR that occurs as a consequence of OVA sensitization/aerosol challenge is abolished in the absence of pulmonary eosinophils. We employed whole body plethysmography to determine if allergen-induced AHR was a concomitant pathophysiological response to the infiltration of the lung and airway lumen by eosinophils. The methacholine dose-response curves (means of single-animal measurements) showed that OVA-sensitized/challenged mice receiving RmCCR3 mAb were not hyperresponsive to methacholine provocation (Fig. 10), in contrast, the cohort of mice receiving nonspecific rat IgG_{2b} showed a methacholine dose response equivalent to OVA-sensitized/challenged wild-type mice, demonstrating the existence of a causative

![Fig. 4. Accumulation of eosinophils to the lung or specific compartments within the pulmonary interstitium is nearly extinguished in response to administration of αCCR3. Eosinophils in the perivascular and peribronchial regions were assessed (n = 5–10 animals/group) by immunocytochemistry using rabbit polyclonal anti-mouse MBP antisera, examining both broad areas of the lung (left, original magnification ×160) and specific regions surrounding individual airways (right, original magnification ×400). A: saline; B: OVA-sensitized/aerosol-challenged wild-type (WT) mice; OVA-sensitized/aerosol-challenged animals administered either ns-IgG (C) or αCCR3 (D) concurrent with the OVA aerosol challenges are shown. Scale bars = 100 μm.](http://ajplung.physiology.org/ by 10.220.32.247 on July 10, 2017)
relationship between allergen-mediated AHR and the presence of pulmonary eosinophils.

DISCUSSION

The link between the recruitment of eosinophils to the lung and the onset/progression of allergic respiratory pathology has remained correlative despite repeated attempts to establish causative mechanisms. For example, anti-IL-5 strategies to ablate eosinophils have inherent ambiguities deriving from observations that IL-5 has potential activities on other cell types (34). The advent of a strategy to specifically ablate eosinophils in the mouse, however, now provides evidence of a causative link. A review of the available evidence shows that the value of this strategy is uniquely a function of the eosinophil specificity of targeting CCR3+ cells without identifiable effects on any other cell types or inflammatory responses: 1) Grimaldi and colleagues (20) demonstrated by FACS that rmCCR3 mAb reactivity was restricted to eosinophils. 2) Administration of rmCCR3 mAb led to the depletion of only eosinophils from circulation with no effects on other leukocytes. This effect was also extended to analyses of peripheral lymphoid tissue such as the spleen, where effects were restricted to eosinophils. 3) Dual systemic/local administration of rmCCR3 mAb to mice during the OVA challenge phase of a sensitization/challenge protocol abolished eosinophils from the airway lumen with no observable effects on other cell types present in BAL fluid. In addition, the administration of rmCCR3 mAb had effects only on lung tissue eosinophils and did not lead to the ablation of any other infiltrating/resident leukocyte. Moreover, unlike observations from OVA-sensitized/challenged CCR3-deficient mice (27), rmCCR3 mAb treatment elicited effects in the lung only on eosinophil numbers and did not modulate pulmonary mast cell/basophil numbers. 4) Administration of rmCCR3 mAb to OVA-treated mice had no effects on the underlying mecha-

![Fig. 7. Production of total IgE and OVA-specific IgG1 was unaffected as a consequence of αCCR3-mediated depletion of eosinophils in OVA-sensitized/challenged mice. Total IgE (A) and OVA-specific IgG1 (B) were assessed in naive and OVA-sensitized/aerosol-challenged animals, as well as OVA-sensitized/aerosol-challenged mice administered either ns-IgG or αCCR3 concurrent with the OVA aerosol challenges. Data represent means ± SE (n = 6–10 animals/group). *Significant difference (P < 0.05) from naive animals.]

![Fig. 8. Expression of Th2 cytokines was unaffected as a consequence of αCCR3-mediated depletion of eosinophils in OVA-sensitized/challenged mice. A: cohorts of mice were killed 12 h following the last OVA aerosol challenge (day 27) to determine BAL levels of IL-4, IL-5, and IL-13 in saline control and OVA-sensitized/aerosol-challenged animals, as well as OVA-sensitized/aerosol-challenged mice administered either ns-IgG or αCCR3 concurrently with the OVA aerosol challenges. Data represent means ± SE (n = 6–10 animals/group). *Significant difference (P < 0.05) from naive animals. B: splenocytes from OVA-sensitized/aerosol-challenged WT and αCCR3-treated mice were cultured in the presence of OVA, and the production of IL-4, IL-5, and IFN-γ was determined by ELISA of the culture supernatants. No significant differences were observed for production of any of the cytokines between either cohort of mice. Values presented are means ± SE of duplicate determinations conducted on 2 separate occasions.]

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nisms leading to the induced Th2 inflammatory responses. In particular, administration of ramCCR3 mAb had no effects on B or T cell activities. 5) Administration of ramCCR3 mAb had no apparent effects on lung structure and did not affect the morbidity/mortality of the mice, suggesting that "structural" cell types in the mouse were unaffected by this depleting antibody.

In addition to the eosinophil specificity of the protocol employed, the ability of this strategy to effectively abolish eosinophils in the BAL and reduce levels in peribronchial/perivascular regions to levels equivalent to saline-challenged control mice is essential to the conclusions drawn about the roles of these cells in allergic respiratory inflammation. Previous studies purporting to dissociate pulmonary pathology from the recruitment of eosinophils, including studies demonstrating that AHR occurs in the absence of a significant airway eosinophilia, have likely overlooked the potential role(s) of eosinophils in these respective model systems. For example, methodologies depleting allergen-induced pulmonary eosinophils through cytokine/chemokine deficiencies also have potential consequences on cell types other than eosinophils. The data presented in other studies (see for example Refs. 8, 9, 25) also do not show that AHR occurs in the absence of an airway eosinophilia. Rather, they show that AHR occurs in mice even though the induced airway eosinophilia had been dramatically reduced (i.e., in each of these studies, eosinophil numbers in the experimental mice are low but elevated relative to control animals). The small numbers of eosinophils present may actually be sufficient to elicit AHR. The final unresolved issue is the relative activation state of infiltrating eosinophils. The eosinophils recruited to the lungs in previous studies noted above, albeit few in number, may all be primed and competent to execute effector functions. Thus, it may not be the specific number of eosinophils recruited to the lung that is critical but their activation state and their location within the lung [i.e., perhaps strategically located small subpopulations of activated eosinophils near airway smooth muscle or pulmonary nerve cells are the actual effector cells recruited to the lungs in these models (see for example Ref. 10)].

The link of eosinophils not only to AHR but also to allergen-mediated goblet cell metaplasia/mucus production implies that activities from this cell type are causative of a wide range of pulmonary pathologies. Similar to observations following administration of anti-IL-5 antibodies (30), OVA-induced mucus production decreased following the selective ablation of pulmonary eosinophils. This observation suggests the existence of both eosinophil-independent and -dependent pathways leading to allergen-mediated goblet cell metaplasia/mucus production. The link of this histopathology with eosinophils, however, is surprising as Th2/Th1 BAL cytokine levels were seemingly unaffected, relative to OVA-treated wild-type mice, in the absence of eosinophils. In addition, a number of previous studies has suggested that such a link does not exist. For example, adoptive transfer studies using OVA T cell receptor transgenic animals and IL-5-deficient mice showed that allergen-induced mucus production can occur in the absence of both IL-5 and an extensive pulmonary eosinophilia (7). These studies suggest that CD4+ T cells and IL-4 receptor expression are each necessary (and possibly sufficient) for aller-

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Fig. 9. Goblet cell metaplasia/mucus production was significantly reduced as a consequence of αCCR3-mediated depletion of eosinophils in OVA-sensitized/challenged mice. Quantitative assessments of airway epithelial mucus content were performed from cohorts representing saline- and OVA-sensitized/aerosol-challenged animals, as well as OVA-sensitized/aerosol-challenged mice administered either ns-IgG or αCCR3 concurrently with the OVA aerosol challenges. Mucus content indexes were derived from 5–10 animals/group and expressed as means ± SE. All evaluations of histopathology were performed in duplicate as independent observer-blinded assessments. *P < 0.05.

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Fig. 10. Airway hyperresponsiveness does not develop in OVA-sensitized/challenged mice as a consequence of αCCR3-mediated depletion of eosinophils. Methacholine dose-response curves resulting from group mean data derived from 4 cohorts of animals (n = 8–16 animals/group), including saline- and OVA-sensitized/aerosol-challenged animals, as well as OVA-sensitized/aerosol-challenged mice administered either ns-IgG or αCCR3 concurrent with the OVA aerosol challenges. Airway responsiveness [enhanced pause (Penh)] in response to increasing doses of nebulized methacholine was assessed by whole body plethysmography. *Significant difference (P < 0.05) between OVA and ns-IgG groups compared with either saline- or αCCR3-treated groups.
gen-induced mucus overproduction (7) with particular focus centering on IL-13 as the relevant cytokine ligand (21). We would suggest that the resolution of the quandary as to whether eosinophils participate in mucous production is simply that these previous studies demonstrated that mucous production was possible in a relative absence of eosinophils, not that eosinophils have no role in this histopathology. Indeed, the mucous production occurring in eosinophil-depleted mice demonstrates that this response, in part, occurs independent of eosinophils. Nonetheless, the absolute magnitude of goblet cell metaplasia/mucus production in the absence of eosinophils was significantly lower, implying that an activity(ies) of eosinophils augments the levels of pathology induced by IL-4/IL-13-dependent mechanisms.

The demonstration that eosinophils contribute to multiple allergen-induced pulmonary pathologies suggests that upon, and/or concomitant with, recruitment to the lung, eosinophils undergo changes resulting in the execution of effector functions. Several lines of evidence implicate IL-4/IL-13 as potential factors mediating mechanisms accounting for the seemingly independent, and yet dependent, behavior of allergic pulmonary pathologies on eosinophil effector functions. IL-4/IL-13 receptor ligand interactions occur on many different cell types in the lung, including T (19) and B lymphocytes (5), mast cells (41), lung endothelial cells (3), lung epithelial cells (42), pulmonary alveolar macrophages (15), and eosinophils (14). Any one of these interactions, particularly events associated with the expression of IL-13, is likely necessary, but not sufficient, to elicit allergic pulmonary pathologies (17). Moreover, the end point levels of observed pathologies are also likely the result of both the linear sum of each pathway’s contribution and also a result of synergistic effects between multiple pathways. For example, IL-4/IL-13-mediated effects on T cells may have downstream consequences on other cell types in the lung contributing to pulmonary pathology. The possibility exists that IL-4/IL-13 receptor ligand interactions directly on eosinophils may signal changes that generally have been described in the literature as “activation.” In this model, IL-4/IL-13 receptor ligand interactions elicit both eosinophil-independent events and the execution of eosinophil effector function(s), the sum of which is responsible for the development of pulmonary pathologies following allergen challenge (43). The immunoregulative capacity of eosinophils themselves may even contribute to this process. Pulmonary eosinophils following allergen challenge have been shown to express GATA-3 resulting in the de novo activation of Th2 cytokine genes (28). Moreover, Bandeira-Melo and colleagues (2) demonstrated that eotaxin, through CCR3-mediated signaling pathways, rapidly mobilizes preformed stores of IL-4 in eosinophils through vesicular transport mechanisms. Although the release of IL-4/IL-13 by eosinophils themselves appears insufficient to significantly affect total BAL cytokine levels, localized release within pulmonary microenvironments may modulate immune responses in areas where eosinophils have concentrated (e.g., peribronchial regions) by autocrine/paracrine regulatory mechanisms.

The provocative consequence of IL-4/IL-13 receptor ligand-mediated events on eosinophils is the identification of a mechanism linking eosinophils with T cell-derived signals. This suggests that the apparent codependence of OVA-induced pathologies on CD4+ T cells is the result of T cell-eosinophil interactions leading to the execution of eosinophil effector functions and, therefore, to the onset/progression of pulmonary pathology. Recent studies demonstrate a potential role of eosinophils as antigen presentation cells (APC) capable of interacting with T cells in the lymph nodes of the lung (40). In addition, MacKenzie and colleagues (36) suggest that this APC function is a critical/prominent effector function of recruited eosinophils. Thus, a simplistic explanation for the lack, or attenuation, of pulmonary pathologies in the absence of eosinophils is that the APC functions of these cells are necessary for the underlying inflammation. However, it is noteworthy that the anti-CCR3-mediated loss of eosinophils from the lung did not have a demonstrable effect on T cell activities, demonstrating that while eosinophils are capable of functioning as APCs, this activity does not appear to be a significant component of the immune responses leading to allergic respiratory inflammation. Instead, “action at a distance” paradigms, in which T cells and eosinophils communicate through secreted cellular signals, are likely mechanisms resulting in the activation of one or both cell types and, in turn, the development of pulmonary pathologies. Interestingly, the lack of phenotypic effects (relative to wild type) in OVA-treated knockout mice deficient of eosinophil secondary granule protein genes [e.g., MBP-1 (13) or EPO (12)] and electron microscopy studies failing to find evidence of eosinophil degranulation in the mouse (37) suggest that the relevant eosinophil effector functions contributing to mucus production and/or AHR do not include release of secondary granule proteins. The implicit conclusion is that eosinophil effector functions are mediated by a previously overlooked mechanism(s) and/or a heretofore unknown pathway(s).

Collectively, observations suggesting that eosinophils are necessary, but not sufficient, to elicit pulmonary pathologies are consistent with most of the available data in the literature. A notable exception is a clinical study of asthma patients using an anti-IL-5 antibody treatment regime, in which it is concluded that eosinophils are not necessary for allergen-mediated pulmonary pathology (31). These data, however, have since been called into question on the basis of methodological limitations (38) and the demonstration that the antibody treatment had little effect on lung tissue eosinophils (16). In addition, the conclusion that eosinophils did not contribute to airway pathologies was also predicated, in part, by defining the origins of disease in terms of single cellular/molecular events (i.e., so-called “all or none” theses of causation), a criterion that no one cell type, or molecule, is likely to fulfill as an explanation for a polygenic syndrome (11) such as asthma. The demonstration in this study that...
eosinophils are required for allergen-induced pulmonary pathologies in the mouse provides evidence of a direct causative relationship. Moreover, the data support an expanded view of eosinophil activities in the lung and suggest that potential interactions with T cells are underlying mechanisms leading to allergic respiratory inflammation and lung dysfunction.

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REFERENCES


27. Humbles AA, Lu B, Friend DS, Okinaga S, Lora J, Al-Garawi A, Martin TR, Gerard NP, and Gerard C. The murine CCR3 receptor regulates both the role of eosinophils and