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The Effect of IL-5 and Eotaxin Expression in the Lung on Eosinophil Trafficking and Degranulation and the Induction of Bronchial Hyperreactivity

Arne W. Mould,* Alistair J. Ramsay,‡ Klaus I. Matthaei,* Ian G. Young,* Marc E. Rothenberg,‡ and Paul S. Foster**

The mechanisms regulating the selective migration and degranulation of eosinophils in the asthmatic lung and the subsequent development of airways hyperreactivity (AHR) have not been fully delineated. In this investigation, we have employed a novel transgenic model to facilitate the dissection of the contributions of IL-5 and/or eotaxin to eosinophil function in the absence of complex tissue signals derived from the allergic lung. Gene transfer of IL-5 and/or eotaxin to the lungs of naive mice induced a pronounced and selective airways eosinophilia, but did not result in eosinophil degranulation or AHR. Airways eosinophilia occurred independently of the induction of a blood eosinophilia, but was markedly augmented by the coexpression of both cytokines and/or by the transient mobilization of eosinophils from the bone marrow by the administration of i.v. IL-5. However, for eosinophil degranulation and AHR to occur, the inhalation of Ag was required in association with IL-5 and eotaxin expression. Investigations in IL-5-deficient mice linked eosinophilia, and not solely IL-5 and eotaxin, with the induction of AHR. Furthermore, eosinophil degranulation and AHR were dependent on CD4+ T cells. Importantly, this investigation shows that IL-5 regulates eosinophilia within the lung as well as in the circulation and also amplifies eotaxin-induced chemotaxis in the airway compartment. Moreover, the interplay between these cytokines, CD4+ T cells, and factors generated by Ag inhalation provides fundamental signals for eosinophil degranulation and the induction of AHR.

Eosinophilic inflammation of the airways plays a critical role in the pathogenesis of asthma (1–3). Clinical and experimental investigations show a strong correlation between the presence of eosinophils and their products in the airways, disease severity, and the development of enhanced bronchial reactivity to spasmogens (airways hyperreactivity (AHR)) (2–7). Once recruited to the lung in response to allergenic stimuli, eosinophils induce pathophysiologic changes to the airways through the secretion of a range of proinflammatory molecules (2, 3). In particular, eosinophils may mediate enhanced bronchial reactivity and damage to the respiratory epithelium by the secretion of major basic protein (MBP) (8–10). In asthma, eosinophils may also localize to the airways mucosa, where they have the potential to prime and amplify the underlying immune responses that predispose to allergic inflammation by the secretion of cytokines (11–16). Although there is accumulating evidence that eosinophils drive the pathogenesis of allergic disease under some circumstances, the mechanisms regulating their selective trafficking and degranulation in the airways have not been fully delineated.

The trafficking of eosinophils to the airways is a complex process that may be regulated by a multiplicity of cytokines, chemokines, adhesion molecules, and lipid mediators (2, 17, 18). Collectively, these molecules form a coordinate network that regulates eosinophil development, release from the bone marrow, accumulation, effector function, and survival in tissues. Once recruited to tissues, eosinophils receive signals that promote degranulation and the secretion of cytokines. Investigations in vitro also indicate the potential role of various cytokines, lipid mediators, and IgE as triggers for the induction of degranulation (3, 19, 20). Of the inflammatory mediators postulated to contribute to the regulation of eosinophil trafficking and degranulation, only IL-5 and eotaxin have been identified to selectively regulate eosinophil function.

Investigations suggest that eotaxin plays an integral role in the baseline homing of eosinophils to mucosal tissues and during the early phase of eosinophil recruitment following allergen challenge (21–25). Eotaxin may also regulate the mobilization of eosinophils and their precursors into the blood (23, 25, 26). IL-5 regulates the growth, differentiation, and activation of eosinophils, and has been strongly implicated in the etiology of asthma (27–32). Studies with IL-5-deficient (IL-5−/−) mice indicate that this cytokine provides an essential signal for the induction of blood and pronounced tissue eosinophilia that is observed during allergic lung inflammation (33). A limited number of studies suggest that eotaxin and IL-5 cooperate to regulate eosinophil trafficking at baseline and during allergic inflammation (34–36). The accumulation of eosinophils into the skin of guinea pigs and mice in response to eotaxin is amplified by the i.v. administration of IL-5 (34, 35). Furthermore, eotaxin-induced recruitment of eosinophils into the skin and lungs is only consistently observed in transgenic mice that constitutively

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2 Abbreviations used in this paper: AHR, airways hyperreactivity; ASM, airways smooth muscle; BALF, bronchoalveolar lavage fluid; HA, hemorrhagin; i.n., intranasal; MBP, major basic protein; TK, thymidine kinase; VV, vaccinia virus.
express elevated levels of IL-5 and have a pronounced blood eosinophilia (36). In addition to regulating eosinophil trafficking, both IL-5 and eotaxin have also been implicated in the mechanism leading to the activation of eosinophils (30, 37). However, the physiological significance of these in vitro degranulation studies to the fundamental processes initiating eosinophil degranulation in the airways and subsequent induction of AHR has not been defined.

In asthma, IL-5 levels are often elevated in the lung and blood, suggesting that this cytokine has the potential to regulate eosinophil function in both compartments (29). Although IL-5 and eotaxin cooperate to selectively regulate tissue eosinophilia, the role of these cytokines in regulating blood and airways eosinophilia, eosinophil degranulation, and AHR when expressed in the lung has not been fully determined. In this investigation, we have employed a transgene approach to selectively identify the roles played by IL-5 and eotaxin in eosinophil recruitment and degranulation and the development of AHR. By gene transfer of eotaxin and/or IL-5 to the airways of naive mice, we have investigated the effect of transient expression of these molecules on the level of blood and airways eosinophils and their activation status in the lung. Furthermore, we determine whether IL-5 and eotaxin provide key signals for the induction of AHR to cholinergic stimuli. In addition, we have also employed our transgene model in naive mice to selectively examine the effect of Ag inhalation on eosinophil degranulation and AHR in the presence of eotaxin and IL-5.

Materials and Methods

Mice

C57BL/6 mice (male, 6–8 wk old) and strain-matched IL-5-deficient mice (38) were supplied by Pathogen Free facilities at the John Curtin School of Medical Research, Australian National University (Canberra, Australia).

Construction of recombinant vaccinia viruses

A recombinant vaccinia virus (VV) containing murine eotaxin cDNA (VV-HA-eotaxin) under the control of the P7.5 VV promoter was generated as previously described (39, 40). Briefly, VV-HA-eotaxin was constructed from VV-HA-PR8, which carries the hemagglutinin (HA) gene of the influenza virus A/PR8/34 and the thymidine kinase (TK) gene of the HSV in the HindIII sites of the J and F regions, respectively. Recombinant vaccinia virus was purified using marker rescue of the TK gene under methotrexate selection. VV-HA-IL-5 and the control virus (VV-HA-TK) were previously constructed (39). Expression of encoded IL-5 (39) and eotaxin (unpublished data (eosinophil-specific migration assays)) by VV-HA-IL-5 and VV-HA-eotaxin was confirmed by specific bioassay in vitro.

Analysis of viral growth in vivo

Mice were given an intranasal (i.n.) inoculum of 1 \times 10^7 PFU of VV-HA-eotaxin, VV-HA-IL-5, or VV-HA-TK, or a co-inoculum of 0.5 \times 10^7 PFU each of VV-HA-eotaxin and VV-HA-IL-5. At 1–7, and 9 days after viral inoculation, mice were sacrificed by cervical dislocation and the lungs removed under sterile conditions. Lung viral titers were determined by the BALF and histology were analyzed. The induction of a late-phase AHR to \beta-methylcholine was determined on day 11 (Fig. 1b). Responses in saline-treated mice were characterized simultaneously.

The effect of eotaxin and/or IL-5 expression on the induction of blood and airways eosinophilia

Mice were treated as described in regime A. In addition, blood smears were taken daily post viral inoculations, and the percentage of eosinophils in the total circulating leukocyte population was determined by differential staining (33). Blood samples were also taken before, and at 30 min i.v. postinjection to confirm the induction of blood eosinophilia by IL-5. Blood eosinophil levels were determined using the method of Discombe (41).

The effect of the coexpression of eotaxin and IL-5 in the airways on eosinophil degranulation and the induction of AHR

VV-HA-eotaxin and VV-HA-IL-5 or VV-HA-TK was administered i.n., and IL-5 or control vehicle was injected i.v., as described in regime A. On day 8, the airways were analyzed for the induction of a late-phase AHR to \beta-methylcholine and for the presence of eosinophils and morphological changes. Leukocyte infiltration into the BALF and the presence of extracellular MBP in BALF supernatants were also determined.

The effect of Ag inhalation on eosinophil degranulation and AHR in the presence of eotaxin and IL-5

Mice were treated as described in regime B. In addition, groups of IL-5−/− mice were also exposed to viral constructs and i.v. treatments, and challenged with saline or OVA.

Determining the role of CD4+ T cells in the induction of eosinophil degranulation and AHR

Mice were treated as described in regime B. In addition, on days 1 and 6, 1 mg of anti-CD4 mAb (clone GK1.5) or of rat isotype control IgG mAb (clone GL113) was injected i.p. We have previously shown that this treatment regime results in the depletion of CD4+ T cells in the spleen and
peribronchial lymph nodes during the course of OVA inhalation and inhibits the development of CD4+ Th2 cell-dependent allergic disease of the lung (42).

**Induction of allergic airways inflammation and AHR**

To compare airways reactivity after IL-5 and eotaxin gene transfer experiments with that which occurs in models of allergic lung disease, mice were sensitized by i.p. injection with 50 μg OVA/1 mg Alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline on days −24 and −12. Non-sensitized mice received 1 mg of Alhydrogel in 0.9% saline (33). From day 1, both groups of mice were aeroallergen challenged (OVA), and AHR was measured as described in regime B. Comparisons were made between controls that were positive (OVA sensitized and aerosol) or negative (saline i.p. and OVA aerosol) for the development of AHR.

**Detection of free MBP in BALF**

BALF was centrifuged (350 × g, 4°C) for 5 min. Aliquots of supernatant were removed and analyzed by immunoblot for the presence of MBP, as previously described (42).

**The analysis of histological sections and leukocyte populations in the blood and BALF**

Transverse sections (~4 mm wide) of lung were cut from the left lobe and fixed in 10% neutral buffered Formalin for a minimum of 24 h. Samples were then processed by the Histology Unit (John Curtin School of Medical Research, Australian National University). Leukocytes in the blood, BALF, and lung were identified by morphological criteria after differential staining with Giemsa-May-Grunwald (33, 42). Routinely, 300–400 cells were counted per slide.

**Measurement of AHR**

AHR was measured with a bronchospasm transducer (Ugo Basile 7020), which was coupled to a Lab Mac/8 analysis station (AdInstruments, Sydney, Australia), as previously described (33). Changes in respiratory flow volume were determined during cumulative i.v. administration of β-methylcholine. The increase in respiratory flow volume provoked by β-methylcholine is represented as a percentage of maximal airways occlusion.

**Materials**

Mouse rIL-5 was expressed and purified from the baculovirus expression system (43), and protein levels were estimated by OD at 280 nm and Bio-Rad (Richmond, CA) protein assay using gamma-globulin as the standard.

**Statistical analysis**

The significance of differences between experimental groups was analyzed using Student’s unpaired t test. Differences in means were considered significant if p < 0.05.

**Results**

**Viral growth and clearance**

The kinetics of viral growth and clearance in the lung were similar for VV-HA-eotaxin and VV-HA-TK when inoculated with 1 × 10^7 PFU (Fig. 2a). Viral titers peaked 4 to 5 days after administration (~10^9 PFU/lung) and subsequently declined to undetectable levels (<10^3 PFU/lung) by day 9. Growth and clearance of VV-HA-eotaxin or VV-HA-TK (Fig. 2a) in the lung were also similar to co-inoculation with VV-HA-eotaxin and VV-HA-IL-5 (0.5 × 10^7 PFU/virus) (Fig. 2b) and to that previously reported for VV-HA-IL-5 (at 1 × 10^7 PFU) (39). Thus, vaccinia growth and clearance from the lung were not affected by the expression of IL-5 or eotaxin, alone or in combination, and the kinetics were similar to the control virus (Fig. 2).

**Expression of eotaxin or IL-5 in the lung induces a pronounced airways eosinophilia that was amplified by the coexpression of these cytokines or i.v. IL-5**

Exposure of the lung to VV-HA-eotaxin or VV-HA-IL-5 or in combination (eotaxin + IL-5) induced a pronounced and selective airways eosinophilia that peaked 6 days after administration (regime A, Fig. 3). Furthermore, VV-HA-eotaxin and VV-HA-IL-5 were equipotent at inducing airways eosinophilia. The level of eosinophils in the BALF in the presence of VV-HA-TK (control) was similar to that observed in nontreated mice (<0.2 × 10^3 eosinophils/ml BALF). Notably, airways eosinophilia induced by eotaxin and/or IL-5 was significantly amplified by the i.v. administration of IL-5 on the day of peak viral titers (Fig. 3). The coexpression of eotaxin and IL-5 in conjunction with i.v. IL-5 treatment induced the most potent and sustained airways eosinophilia. The numbers of neutrophils, lymphocytes, and macrophages in the BALF were not significantly different between groups treated with viral constructs (results not shown; moreover, the levels of these cells in BALF from VV-HA-eotaxin, VV-HA-IL-5, VV-HA-TK, or coexpression studies on day 6 were not significantly different from those shown for day 8, 48 h later (see Fig. 5a)). Intravenous IL-5 induces a pronounced blood eosinophilia that peaks rapidly (30 min) and declines to baseline levels over 5 h (results not
Expression of eotaxin and IL-5 in the lung promotes a selective airways eosinophilia. In initial studies, the effect of IL-5 or eotaxin expression alone and in combination on the number of eosinophils in the airways in the absence and presence of a peripheral eosinophilia was investigated (regime A). VV-HA-eotaxin (eotaxin), VV-HA-IL-5 (IL-5) (alone or in combination with eotaxin), or VV-HA-TK (control) was administered i.n., and 4 days later IL-5 or control vehicle was injected i.v. (Fig. 1A). Expression of eotaxin and IL-5 in the airways did not promote a peripheral eosinophilia (result not shown). The levels of eosinophils and other leukocytes in BALF were then quantified 24 h after i.v. injections on day 6. Results only show eosinophil numbers. The levels of other leukocytes in the BALF were not significantly different between groups of mice that had been treated with viral constructs and are similar to the levels shown for the respective groups on day 8 (Fig. 5A). Data represent the mean eosinophils/ml BALF ± SEM of groups of five mice. *, p < 0.01 when compared with eotaxin or IL-5 alone or in combination. **, p < 0.05 when compared with eotaxin or IL-5, and ***, p < 0.01 when compared with eotaxin + IL-5 with control i.v.

Notably, the expression of IL-5 or eotaxin or both cytokines in the lung failed to significantly increase blood eosinophil levels (results not shown).

Histological examination of lung sections showed a selective recruitment of eosinophils to the peribronchial regions in VV-HA-eotaxin- and VV-HA-IL-5-treated airways (Fig. 4B). Eosinophils were also observed migrating through the vasculature (not shown). Similar results were obtained with the expression of eotaxin or IL-5 alone (results not shown). Eosinophils in the peribronchial regions were predominantly localized to areas associated with the airways smooth muscle (ASM) layer. Eosinophilia induced by eotaxin and IL-5 did not result in significant morphological changes to the respiratory epithelium (Table I). As a control for the development of these features of allergic disease, responses after VV-HA-eotaxin and VV-HA-IL-5 treatment were compared with those observed in an established model of allergic airways disease. Intratracheal sensitization and subsequent induction of allergic airways disease by OVA inhalation (BALF inflammatory cell numbers not shown) resulted in the development of marked AHR (Fig. 5B, positive control) and the detection of a strong signal for MBP on immunoblots of cell-free BALF (Table I). Furthermore, baseline airways reactivity in VV-HA-eotaxin- and VV-HA-IL-5-treated lung was similar to that observed in the absence of airways eosinophilia in VV-HA-TK-treated and negative control mice. Thus, although eotaxin and IL-5 expression regulated eosinophilia within the lung compartment, signals elicited by these cytokines did not induce eosinophil degranulation or AHR. Furthermore, exposure of the airways to viral constructs did not affect baseline airways reactivity.

Expression of eotaxin and IL-5 in the airways does not induce eosinophil degranulation or the induction of AHR

Although coexpression of eotaxin and IL-5 selectively induced a significant BALF and airways eosinophilia (Fig. 3, day 6, and Fig. 5A, day 8), they did not induce a late-phase AHR to the cholinergic agonist β-methylcholine (Fig. 5B, only data for day 8 shown). Furthermore, no MBP was detected in cell-free extracts of BALF taken from the airways expressing eotaxin and IL-5 on day 6 or 8 (Table I). As a control for the development of these features of allergic disease, responses after VV-HA-eotaxin and VV-HA-IL-5 treatment were compared with those observed in an established model of allergic airways disease. Intratracheal sensitization and subsequent induction of allergic airways disease by OVA inhalation (BALF inflammatory cell numbers not shown) resulted in the development of marked AHR (Fig. 5B, positive control) and the detection of a strong signal for MBP on immunoblots of cell-free BALF (Table I). Furthermore, baseline airways reactivity in VV-HA-eotaxin- and VV-HA-IL-5-treated lung was similar to that observed in the absence of airways eosinophilia in VV-HA-TK-treated and negative control mice. Thus, although eotaxin and IL-5 expression regulated eosinophilia within the lung compartment, signals elicited by these cytokines did not induce eosinophil degranulation or AHR. Furthermore, exposure of the airways to viral constructs did not affect baseline airways reactivity.

Expression of eotaxin and IL-5 in the lung induces enhanced AHR to β-methylcholine following inhalation of OVA

Next, the airways of naïve mice expressing eotaxin and IL-5 (and control groups) were exposed to OVA or saline to determine whether signals associated with Ag inhalation could activate airways eosinophils and induce immunopathological responses associated with allergic disease of the lung (regime B). OVA inhalation...
numbers in the BALF were significantly higher in both of the groups treated with viral constructs (p < 0.05) following OVA inhalation, when compared with saline-exposed controls (Fig. 6a, day 11). Strikingly, in the airways expressing eotaxin and IL-5, OVA inhalation resulted in the induction of eosinophil degranulation (free MBP in the BALF) (Table I) and AHR to β-methylcholine (Fig. 6b). The eotaxin/IL-5-regulated airways eosinophilia did not predispose to the development of these immunopathological features of allergy in the saline-treated group (Fig. 6 and Table I) or with VV-HA-IL-5 and VV-HA-eotaxin alone (Fig. 5). Similarly, these features were not observed in the airways exposed to VV-HA-TK and OVA (Fig. 6 and Table I). Notably, AHR in the model of allergic lung disease (positive control) that was induced by i.p. sensitization and aerosol challenge with OVA was more pronounced in comparison with that observed in the airways of naïve mice when eotaxin and IL-5 were expressed in the presence of OVA (Fig. 6b). OVA inhalation and/or viral constructs (Fig. 6b) did not effect basal AHR. Although eotaxin and IL-5 expression induced eosinophil degranulation and AHR in the presence of OVA, no pronounced morphological changes to the airways were observed (histology not shown). Furthermore, the eosinophils that were present in the airways were consistently and predominantly localized to regions associated with ASM. These results strongly suggest that signals elicited during Ag inhalation in the lung can result in degranulation of airways eosinophils and predispose to the development of enhanced bronchial reactivity.

IL-5/− mice have very low levels of circulating and tissue-dwelling eosinophils, and the expression of IL-5 alone in the lung does not result in airways eosinophilia (33, 44). Thus, we employed IL-5/− mice to determine whether IL-5 plus eotaxin expression in the lung was sufficient to induce AHR independently of eosinophils. IL-5/− mice (six per group) were treated with VV-HA-IL-5 and VV-HA-eotaxin or VV-HA-TK and exposed to OVA or saline. Expression of eotaxin and IL-5 in the airways did not induce a significant airways eosinophilia (<1% of total BALF cells) or reestablish normal circulating levels of eosinophils (<1% of total circulating leukocytes). In addition, AHR was not induced after OVA inhalation (results not shown). Thus, the presence of airways eosinophils and not solely IL-5 and eotaxin was associated with the development of AHR during Ag inhalation.

Eosinophil degranulation and the development of AHR in the lungs expressing eotaxin and IL-5 in the presence of OVA are CD4+ T cell dependent

The development of the immunopathological processes associated with allergic airways disease has been shown to be exclusively regulated by CD4+ T cells in mouse models (42). To determine the role of CD4+ T cells in the induction of AHR and eosinophil degranulation in the presence of OVA inhalation and VV-HA-IL-5

Table I. Correlation of AHR with the detection of MBP in BALF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-5 + Eotaxin</th>
<th>IL-5 + Eotaxin + OVA</th>
<th>IL-5 + Eotaxin + Anti-CD4</th>
<th>Allergic Lung Disease</th>
<th>Eosinophil Supernatants</th>
<th>T Cell Supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of MBP</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Airways hyperreactivity</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>-</td>
</tr>
</tbody>
</table>

* Cell-free BALF samples were assayed for the presence of MBP by immunoblot analysis as described in Material and Methods. MBP was detected as a strong positive signal (+) or at background levels (−). Results represent analysis of four to five samples per experimental group. MBP levels were determined after exposure of the airways to VV-HA-IL-5 (IL-5) + VV-HA-eotaxin (eotaxin) on day 6 or 8 (regime A) or on day 9 after OVA or saline treatments or OVA + anti-CD4 treatments (regime B). MBP was detected on day 9 and correlated with the development of airways hyperreactivity on day 11. BALF MBP levels were also assayed after VV-HA-TK + OVA treatment (not detected) and in eosinophil and T cell supernatants or in the model of allergic lung disease.
and VV-HA-eotaxin, anti-CD4 mAb or isotype control was administered i.p. Anti-CD4 mAb (or isotype control) treatment did not affect individual leukocyte levels in the BALF (Fig. 7a). However, anti-CD4 mAb (but not isotype control) inhibited eosinophil degranulation (Table I) and the development of AHR (Fig. 7b). Numbers of leukocytes in the BALF after VV-HA-TK and OVA treatments in the presence of anti-CD4 mAb or isotype control were similar (results not shown) and not significantly different from the data shown for VV-HA-TK + OVA (Fig. 7a). AHR was also measured after VV-HA-TK and OVA treatment in the presence of anti-CD4 (GK1.5) (Fig. 7b) or control Ab (GL113) (not shown), and responses were not significantly different between these groups.

Discussion

In this investigation, we have identified key mechanisms for the regulation of eosinophil recruitment and degranulation in the airways and for the development of AHR. Expression of IL-5 and/or eotaxin in the lung induced a pronounced and selective airways eosinophilia. Furthermore, tissue signals elicited by IL-5 and eotaxin in the lung induced a pronounced and selective airways eosinophilia. AHR was also measured after VV-HA-TK and OVA treatment in the presence of anti-CD4 (GK1.5) (Fig. 7b) or control Ab (GL113) (not shown), and responses were not significantly different between these groups.

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FIGURE 6. Ag inhalation predisposes to AHR in the presence of airways mucosal eosinophils. To determine whether signals associated with Ag inhalation in the airways were required with eosinophils to induce AHR, OVA or saline was administered by aerosol, as described in regime B (Fig. 1b). VV-HA-eotaxin and VV-HA-IL-5 (eotaxin + IL-5) or VV-HA-TK (control) was administered i.n., and 4 days later (day 8) IL-5 or control vehicle was injected i.v. Thus, the temporal arrangement between the administration of viral vectors and i.v. delivery of IL-5 was similar to previous experiments (Figs. 5 and 1b). On day 11 (48 h after the peak eosinophil response), leukocyte levels in BALF (a), and late-phase airways reactivity to i.v. β-methacholine (b) were measured. Airways reactivity responses obtained after IL-5 and eotaxin gene transfer experiments were also compared with responses obtained in an established model of allergic lung disease (positive (OVA sensitized and aerosoled) or negative (saline i.p. and OVA aerosoled) controls for AHR). Data represent mean cells/ml BALF (a), and the mean percentage of airway occlusion ± SEM from groups of six mice (b). *, p < 0.05 when compared with groups that were exposed to saline aerosol (a); and *, p < 0.05, and **, p < 0.01 when compared with VV-HA-TK + OVA aerosol (control + OVA) and VV-HA-IL-5 + VV-HA-eotaxin + saline aerosol, or negative control (saline i.p. and OVA aerosoled) groups (b).

FIGURE 7. CD4+ T cells regulate Ag-induced AHR in the presence of airways eosinophils. To determine whether CD4+ T cells regulate Ag-induced AHR in the presence of airways eosinophils, anti-CD4 mAb (GK1.5) or isotype control mAb (GL113) was administered on days 1 and 6 of the regime B (Fig. 1b). VV-HA-eotaxin and VV-HA-IL-5 (eotaxin + IL-5) or VV-HA-TK (control) was administered i.n., and IL-5 was injected i.v. On day 11 (48 h after the peak eosinophil response), leukocyte levels in BALF after treatment with eotaxin + IL-5 + OVA in the presence of GK1.5 or GL113 were measured. Numbers of leukocytes in the BALF after VV-HA-TK and OVA treatments in the presence of GK1.5 or GL113 were similar to each other (not shown) and not significantly different from the data shown for VV-HA-TK + OVA (control + OVA) in Fig. 5a. b, Airways responses were measured after treatment with eotaxin + IL-5 and OVA in the presence of GK1.5 or GL113. Responses after VV-HA-TK and OVA treatments in the presence of GK1.5 (control + OVA + GK1.5) or GL113 (not shown) were not significantly different. Data represent mean cells/ml BALF (a), and the mean percentage of airway occlusion ± SEM from groups of six mice (b). a, No significant difference in the numbers of leukocytes in the respective populations between GK1.5 and GL113 treatments in the presence of IL-5 + eotaxin and OVA. b, *, p < 0.05, and **, p < 0.01 when compared with eotaxin + IL-5 + OVA + GL113. Control + OVA + GK1.5 and control + OVA + GL113 (not shown) and negative control (not shown) were not significantly different.
induce a significant blood eosinophilia (circulating numbers did not rise above homeostatic levels), although i.v. delivery of recombiant or VV-encoded IL-5 induced a rapid and pronounced blood eosinophilia with a subsequent decrease in bone marrow levels of this leukocyte (results not shown and (34)). The failure of eotaxin and IL-5 to induce a blood eosinophilia when expressed in the lung suggests that there is a substantial pool of eosinophils in the circulation under homeostatic conditions that have the potential to be recruited to the lung in response to specific chemotactic stimuli. Collectively, our results suggest that two mechanisms may operate to regulate eosinophilia, one within tissue compartments and the other at the level of the bone marrow. Importantly, like eotaxin (23, 25, 26), IL-5 is a central mediator of both processes.

When compartmentalized to tissues, the role of IL-5 in eosinophil chemotaxis is controversial (22, 24, 25, 33, 34, 45). In particular, after gene transfer of IL-5 by adenoviral vectors to IL-5−/− mice, it was postulated that IL-5 in the circulation and not lung production of this cytokine was essential for promoting blood and airways eosinophilia after sensitization and aerosol allergen challenge (44). The potent chemotactic signal elicited by IL-5 and eotaxin in our investigation also failed to induce a pronounced airways or blood eosinophilia in naive IL-5−/− (but not in wild-type) mice. However, we have previously shown that transient expression of IL-5 in the lungs of allergic mice deficient in this cytokine reconstitutes both blood and airways eosinophilia after allergen inhalation (33). This and other investigations indicate that additional factors released from the site of Ag challenge are required to amplify the IL-5 signal to generate both airways eosinophilia and allergic responses (33, 34, 44). Attempts to induce eosinophil trafficking in IL-5−/− mice are compounded by the extremely low circulating and tissue levels of eosinophils. It is likely that IL-5 expression in the lung of IL-5−/− mice with adenoviral or VV vectors was not sufficient to reconstitute basal levels of eosinophils and thus promote trafficking of this leukocyte to the airways (44 and this study). Furthermore, it is possible that other factors (such as eotaxin) within the lung that are required to augment the IL-5 signal for the induction of eosinophilia were not sufficiently induced by the sensitization and Ag inhalation protocol employed (44). Our data show that when expressed in the lung of wild-type mice, IL-5 elicits a chemotactic signal (directed migration to the airways) for eosinophils and that this response occurs in the absence of a peripheral blood eosinophilia. Thus, IL-5 operates mechanisms within the lung and in the circulation to regulate eosinophilia, and these mechanisms may potentially operate in asthma (29). Furthermore, eotaxin and IL-5 act in synergy to specifically coordinate blood and tissue eosinophilia (26, 34).

Although eotaxin and IL-5 selectively regulate eosinophil migration, the signals elicited by these cytokines failed to promote the release of MBP, predispose to morphological changes of the airways wall, or enhance airways reactivity to cholinergic stimuli. These results are in marked contrast to the constitutive overexpression of IL-5 in the airways of transgenic mice in which morphological changes to the respiratory epithelium and AHR were observed (46). Sustained exposure of eosinophils to IL-5 induces degranulation, and this process may underlie the pathophysiological changes observed in the airways of these and other studies performed in IL-5 transgenic mice (46, 47). Our model of airways eosinophilia was designed to mimic the transient expression of IL-5 and eotaxin that is observed after allergen inhalation and acute exacerbation of asthma and to avoid the pathophysiological features associated with chronic exposure to these cytokines. Our results support data that eosinophils alone in the airways are not sufficient to induce AHR (48, 49) and suggest that other factors linked with allergic inflammation in association with IL-5 and eotaxin trigger degranulation and AHR.

To determine whether factors elicited by Ag inhalation in the lung predisposed to eosinophil degranulation and AHR, we exposed the airways of naive mice to OVA in the presence of an airways eosinophilia induced by eotaxin and IL-5. Strikingly, immunological processes associated with Ag inhalation resulted in signals that induced eosinophil degranulation (release of MBP) and enhanced airways reactivity to cholinergic stimuli. Importantly, OVA inhalation alone did not induce a blood or airways eosinophilia or significantly amplify eosinophil numbers in these compartments in the presence of IL-5 and eotaxin; thus, induction of these immunopathological features of allergy was only associated with airways eosinophils and IL-5 and eotaxin expression. Notably, expression of IL-5 and eotaxin in the airways of naive IL-5−/− mice failed to promote airways eosinophilia or AHR in response to Ag inhalation. These results strongly suggest that the eosinophil and not these cytokines alone predisposed to the development of AHR. In asthmatics, eosinophils are often resident in the airways mucosa even at quiescent periods, and their contribution to the induction of the asthma after allergen inhalation is unknown (1, 3). Our data suggest that latent eosinophils that are localized to the airways wall can become activated after Ag provocation and mediate AHR.

Notably, the mechanism for the induction of eosinophil degranulation and AHR was dependent on CD4+ T cells. Furthermore, we have preliminary data that suggest OVA inhalation does not predispose to eosinophil degranulation or AHR in MHC-II-deficient mice after IL-5 and eotaxin gene transfer (not shown). Thus, our results suggest that Ag uptake and processing by professional APC in the lung result in the expansion of CD4+ T cells, which subsequently trigger airways eosinophils to degranulate. The mechanism for the induction of CD4+ T cell-mediated AHR in this model is yet to be defined. However, a direct correlation between the presence of MBP in the BALF, localization of eosinophils to the ASM band, and the induction of AHR was observed. Notably, in our investigation, AHR occurred independently of pronounced morphological changes to the respiratory epithelium. Thus, MBP may induce AHR by directly acting on smooth muscle as well as through its cytotoxic properties upon the respiratory epithelium (9, 10, 50). It is tempting to speculate that IL-13 liberated from CD4+ T cells may play a key role in eosinophil activation and/or the induction of AHR (51, 52).

Although it is likely that AHR was mediated through CD4+ T cell-regulated eosinophil degranulation, it should be noted that there is potential for bidirectional signaling between these two leukocytes. Eosinophils express MHC class II molecules (53, 54) and secrete a range of proinflammatory cytokines and chemokines that are CD4+ T cell growth factors and chemoattractants (1–3, 55). GM-CSF expression in the lung and the subsequent recruitment of eosinophils to the airways have also been shown to increase local Ag-presenting capacity to OVA inhalation in naive mice and promote T cell-mediated features of allergy (56). It should be noted that depletion of CD4+ T cells may also have complex effects in terms of both immune responsiveness as well as eosinophils activation. Importantly, we show that CD4+ T cells play a central role in the degranulation of airways eosinophils. Furthermore, CD4+ T cells and eosinophils may collaborate to directly modulate AHR.

In this investigation, we provide new insights into the mechanisms that regulate the selective recruitment and degranulation of airways eosinophils and induce the development of AHR. Collectively, our results show that IL-5 and eotaxin act in synergy to provide elemental signals within the lung for the recruitment of eosinophils to the airways. Importantly, IL-5 not only regulates...
eosinophil migration from the bone marrow, but also eosinophilia within the lung. Moreover, we demonstrate that CD4+ T cells play a key role in the release of MBP from eosinophils and in the subsequent induction of AHR during Ag inhalation. Targeting both IL-5- and eotaxin-regulated eosinophilia in the lung and the bone marrow compartments may be required to effectively reverse chronic lesions and AHR in asthma.

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References


