

Eotaxin-1-Deficient Mice Develop Airway Eosinophilia and Airway Hyperresponsiveness

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Key Words

Eotaxin · Airway hyperresponsiveness · Airway eosinophilia

Abstract

Background: The accumulation of eosinophils in the lung is a hallmark of asthma. In addition to cytokines such as IL-5 which are essential, chemokines have been implicated in the recruitment of eosinophils to the airway. In particular, eotaxin has been shown to be a selective and potent eosinophil chemoattractant, important in the pathogenesis of allergic disease. The goal of the present study was to define the role of eotaxin-1 in the development of allergen-induced eosinophilic airway inflammation and airway hyperresponsiveness (AHR) to inhaled methacholine (MCh). **Methods:** Eotaxin-1-deficient mice were sensitized and exposed to a single challenge with allergen. Airway function and airway and tissue as well as peripheral blood and bone marrow eosinophilia were examined 18 and 48 h after the last challenge. **Results:** Following allergen sensitization and challenge, eotaxin-1-deficient mice developed levels of AHR to inhaled MCh at 18 and 48 h comparable to controls. Further, levels of bronchoalveolar lavage (BAL) and tissue eosinophilia at the same time points were comparable in the two strains of mice. Tissue eosinophilia, assessed by quantitating major basic protein staining cells, preceded BAL eosinophilia in a similar manner. Bone marrow and peripheral blood eosinophilia were unimpaired in deficient mice.

Conclusion: The results demonstrate that the major eotaxin, eotaxin-1 is not essential for the development of airway eosinophilia or AHR, implying that other chemokines, alone or in combination, can overcome this deficiency.

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Introduction

Clinical investigations have identified the eosinophil as a major infiltrating cell in the airways of allergic asthmatics, and is thought to be a major effector cell in the pathogenesis of allergen-induced airway hyperresponsiveness (AHR) [1, 2]. In particular, the release of cytotoxic granule proteins such as major basic protein (MBP), which can damage the airway epithelium are thought to contribute to this phenomenon [3]. A number of murine studies also support the hypothesis that increased levels of eosinophils in the airway underlie the pathogenesis of AHR [4–6].

Considerable effort has focused on the mechanisms involved in the trafficking of eosinophils to the lung. This appears to be a complex process regulated by a diverse array of cytokines, chemokines, adhesion molecules and lipid mediators [7]. The cytokine IL-5 is known to regulate the growth, differentiation, activation and survival of eosinophils, and its importance in the development of allergic airway eosinophilia has been highlighted using IL-5-deficient mice or antibody directed against IL-5 [4, 6, 8, 9]. Al-

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ternately, numerous mediators have been identified as chemoattractants for eosinophils, including lipid mediators such as platelet-activating factor and leukotrienes, and more recently identified C-C chemokines, including monocyte chemotactic proteins (MCP-2, -3, -4), regulated on activation, normal T cells expressed and secreted (RANTES) protein, macrophage inflammatory protein (MIP)-1 α , eotaxin-1, eotaxin-2, and recently, eotaxin-3 [10–14].

Eotaxin-1 has been distinguished from the other C-C chemokines as being largely an eosinophil-selective chemokine. Eotaxin-2 whilst only having 39% homology with eotaxin-1, acts primarily on eosinophils as well [15]. More recently eotaxin-3 was described and appears to be more closely related to eotaxin-2 [13, 14]. The selectivity of eotaxin-1, eotaxin-2 and eotaxin-3 arises from their specific interaction with the C-C chemokine receptor-3 (CCR3), expressed on eosinophils and basophils [13–19]. Eotaxin-1 was initially identified as the major eosinophilic chemoattractant in a guinea-pig model of allergen-induced lung eosinophilia [20]. Eotaxin-1 has since been identified in humans and mice and expression is increased in the lung following allergen challenge in animals and asthmatics [19, 21–28]. Moreover, local administration of eotaxin-1 in vivo either in the lung or skin is a potent inducer of tissue eosinophilia, which interestingly is enhanced by the presence of IL-5 [22, 29, 30]. Eotaxin-1 may have other biologically relevant properties, including the activation of eosinophils, resulting in release of granule proteins and superoxide generation [31, 32]. In addition, eotaxin-1 has also been demonstrated to be a potent chemoattractant of human basophils in vitro [33]. Furthermore, eotaxin-1 is also chemotactic for Th2 cells which are now recognized as expressing the CCR3 receptor [34, 35].

Studies employing targeted disruption of the eotaxin gene or antibody against eotaxin have provided varying results. Eotaxin-neutralizing antibody studies have implicated eotaxin in the recruitment of eosinophils to the lung and the development of AHR following multiple allergen challenges [31, 36]. Interestingly, when a single allergen provocation was used, eotaxin antibody was ineffective [31]. Alternatively, using eotaxin gene-deficient animals, one study demonstrated a reduction in the early eosinophil recruitment to the lung while a second study demonstrated no effect on allergen-induced airway eosinophilia [37, 38]. In these latter two studies the influence of eotaxin deficiency on AHR was not determined. In the present study using eotaxin-1-deficient mice, we have addressed the role of eotaxin not only in the development of airway eosinophilia, but also in the subsequent development of AHR.

Materials and Methods

Animals

Male and female eotaxin-1-deficient (–/–) and eotaxin-1-sufficient mice were utilized and characterized as described previously [37]. They were kindly provided by Dr. M. Rothenberg (Cincinnati, Ohio). The mice were maintained on ovalbumin (OVA)-free diets. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Sensitization and Airway Challenge

Groups of mice, 10–12 weeks of age, were sensitized by intraperitoneal (i.p.) injection of 20 μ g OVA (Grade V, Sigma Chemical Co., St. Louis, Mo.) emulsified in 2.25 mg aluminum hydroxide (Alum-Imuject, Pierce, Rockford, Ill.) in a total volume of 100 μ l on days 1 and 14. Mice were challenged (30 min) via the airways with OVA (3% in saline) on day 28 using ultrasonic nebulization (AeroSonic ultrasonic, nebulizer, De Vilbiss, Sommerset, Pa.). Control mice groups received OVA challenge alone. 18 or 48 h after OVA challenge, AHR was assessed and tissues obtained for further analysis.

Determination of Airway Responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine (MCh). Anesthetized (pentobarbital sodium, i.p., 70–90 mg/kg), tracheostomized (stainless steel cannula, 18G) mice were mechanically ventilated and lung function was assessed using methods described by Takeda et al. [39]. MCh aerosol was administered for 10 s (60 breaths/min, 500 μ l tidal volume) in increasing concentrations (3–50 mg/ml). Maximum values of lung resistance (RL) and minimum values of dynamic compliance (C_{dyn}) were taken and expressed as a percentage change from baseline following saline aerosol.

Bronchoalveolar Lavage Cell Counts

Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with Hank's balanced salt solution (HBSS, 1 $\times$ 1 ml, 37°C). Total leukocyte numbers were measured (Coulter Counter, Coulter Corp., Hialeah, Fla.). Differential cell counts were performed by counting at least 200 cells on cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon Ltd., Runcorn, Cheshire, UK), stained with Leukostat (Fisher Diagnostics) and differentiated by standard hematological procedures.

Blood Cell Counts

Blood samples were collected and blood smears prepared. Total white blood cell number was determined in peripheral blood after red blood cell lysis. Eosinophil numbers were determined by counting at least 200 cells stained with Leukostat.

Bone Marrow Cell Counts

Bone marrow cells were collected from one femur of each mouse. Briefly, the femur was dissected from the animal, and the ends of the femur cut. A 25G needle was inserted into one end of the femur, and flushed out with HBSS (2 ml). Total leukocyte numbers were measured. Eosinophil counts were performed by counting at least 200 cells on cytocentrifuged preparations, stained with Leukostat.

Immunohistochemistry

Lungs were fixed by inflation (1 ml) and immersion in 10% formalin. Cells containing eosinophilic major basic protein (MBP) were identified by immunohistochemical staining as previously described using rabbit anti-mouse MBP (kindly provided by Dr. J.J. Lee, Mayo Clinic, Scottsdale, Ariz.) [4]. The slides were examined in a blinded fashion with a Nikon microscope equipped with a fluorescein filter system. Numbers of eosinophils in the tissues were evaluated using the IPLab2 software (Signal Analytics, Vienna, Va.) for the Macintosh counting 6–8 different fields per animal. Data are expressed as the number of eosinophils per field (counts/field).

Statistical Analysis

Analysis of variance (ANOVA) was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by Tukey-Kramer HSD test. *p* Values for significance were set to 0.05. Values for all measurements are expressed as the mean $\pm$ standard error of the mean (SEM).

Results

Eotaxin-1^{-/-} Mice Develop AHR

To determine the effect of eotaxin deficiency on the development of AHR, OVA-sensitized and non-sensitized, eotaxin-sufficient (eotaxin-1^{+/+}) and eotaxin-deficient (eotaxin-1^{-/-}) mice were challenged with an aerosol of OVA. Either 18 or 48 h after allergen provocation, airway responsiveness (RL, C_{dyn}) to aerosolized MCh was measured. Baseline RL and C_{dyn} measurements at 18 h following saline challenge of eotaxin-1^{+/+} mice receiving OVA challenge alone were 0.63 ± 0.13 cm H₂O/ml/s and 0.047 ± 0.005 cm H₂O/ml respectively, and did not differ significantly (*p* > 0.05) across any of the groups (non-sensitized vs. sensitized; eotaxin-1^{+/+} vs. eotaxin-1^{-/-}) at either 18 or 48 h. Following OVA sensitization and challenge, both eotaxin-1^{+/+} and eotaxin-1^{-/-} mice developed a similar significant AHR to aerosolized MCh at 18 h, when compared with mice that received OVA challenge alone (fig. 1a, b). The increase in airway responsiveness in eotaxin-1^{+/+} and eotaxin-1^{-/-} OVA-sensitized/challenged mice was similarly maintained at 48 h (fig. 1c, d). These data suggest that eotaxin-1 was not required for the development of allergic AHR in this model.

Eotaxin-1^{-/-} Mice Develop Blood and Bone Marrow Eosinophilia

Total cell number and eosinophil number were determined in the bone marrow and blood of eotaxin-1^{+/+} and eotaxin-1^{-/-} mice receiving OVA challenge alone and of OVA-sensitized/challenged mice (fig. 2). Total leukocyte number at 18 h in eotaxin-1^{+/+} mice receiving OVA challenge alone was $5.1 \pm 1.6 \times 10^6$ /femur and did not differ

from any of the groups at either 18 or 48 h. Similarly, bone marrow eosinophil numbers were essentially the same across all groups (fig. 2a). However, it was interesting to note that at both 18 and 48 h, eosinophil numbers in eotaxin-1^{-/-} mice receiving OVA challenge alone were slightly higher than in time-matched eotaxin-1^{+/+} mice receiving OVA challenge alone.

Furthermore, following OVA sensitization and challenge, a slight increase in bone marrow eosinophil number was observed in both eotaxin-1^{+/+} and eotaxin-1^{-/-} mice at both 18 and 48 h, when compared with mice receiving OVA challenge alone.

Total blood leukocyte number at 18 h in eotaxin-1^{+/+} mice receiving OVA challenge alone was $5.8 \pm 1.6 \times 10^6$ /ml and did not differ from any of the groups at either 18 or 48 h. Blood eosinophil numbers were slightly, although not significantly, elevated in both eotaxin-1^{+/+} and eotaxin-1^{-/-} OVA-sensitized/challenged mice, when compared with time-matched eotaxin-1^{+/+} and eotaxin-1^{-/-} mice receiving OVA challenge alone (fig. 2b).

Eotaxin-1^{-/-} Mice Develop Airway Eosinophilia

The number and type of inflammatory cells in the broncho-alveolar lavage fluid (BALF) of eotaxin-1^{+/+} and eotaxin-1^{-/-} mice receiving OVA challenge alone and of OVA-sensitized/challenged mice was assessed (fig. 3). Total cell numbers recovered in BALF were essentially the same in all groups of mice at both 18 and 48 h (fig. 3a). The inflammatory cell type recovered in the BALF of eotaxin-1^{+/+} and eotaxin-1^{-/-} mice receiving OVA challenge alone consisted almost entirely of macrophages at 18 and 48 h (fig. 3b). Following OVA-sensitization and challenge of eotaxin-1^{+/+} and eotaxin-1^{-/-} mice, a significant and similar BALF neutrophilia was observed at 18 h, which had subsided by 48 h (fig. 3d). Although, neither eotaxin-1^{+/+} nor eotaxin-1^{-/-} OVA-sensitized/challenged mice developed a significant BALF eosinophilia at 18 h after challenge, both groups of mice developed a similar significant BALF eosinophilia by 48 h (fig. 3e).

Eosinophilic inflammation was further assessed in airway tissues using an antibody directed against MBP. Eotaxin-1^{+/+} and eotaxin-1^{-/-} mice receiving OVA challenge alone demonstrated little tissue eosinophilia (fig. 4). OVA sensitization and challenge of eotaxin-1^{+/+} and eotaxin-1^{-/-} mice resulted in a significant and similar tissue eosinophilia at 18 h, which was increased at 48 h (fig. 4).

Fig. 1. Eotaxin-1^{-/-} mice develop AHR following OVA sensitization and challenge. Airway reactivity (lung resistance, RL; dynamic compliance, Cdyn) to increasing concentrations of aerosolized MCh was measured at 18 h (**a, b**) and 48 h (**c, d**) in eotaxin-1^{-/-} mice and eotaxin-1^{+/+} mice following OVA challenge alone (challenged), and in OVA-sensitized and challenged (sensitized/challenged) mice. Data represent the mean $\pm$ SEM (n = 5–7 per group). * Significant difference (p < 0.05) between challenged alone and sensitized and challenged groups.

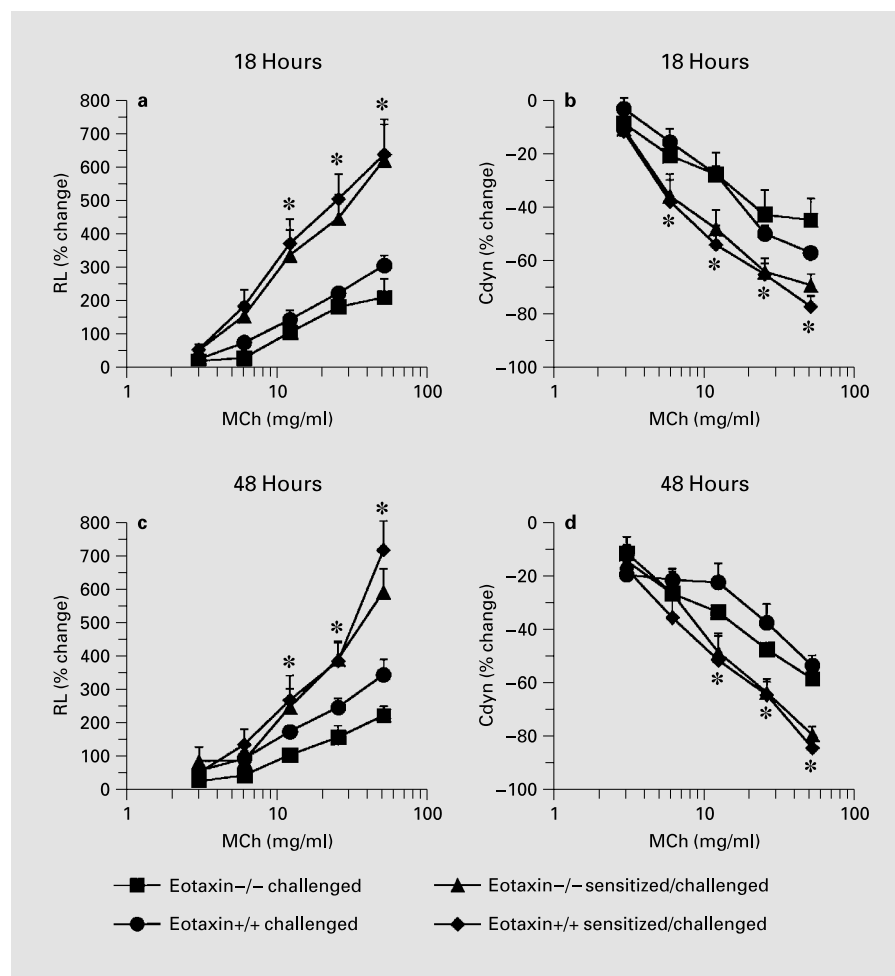
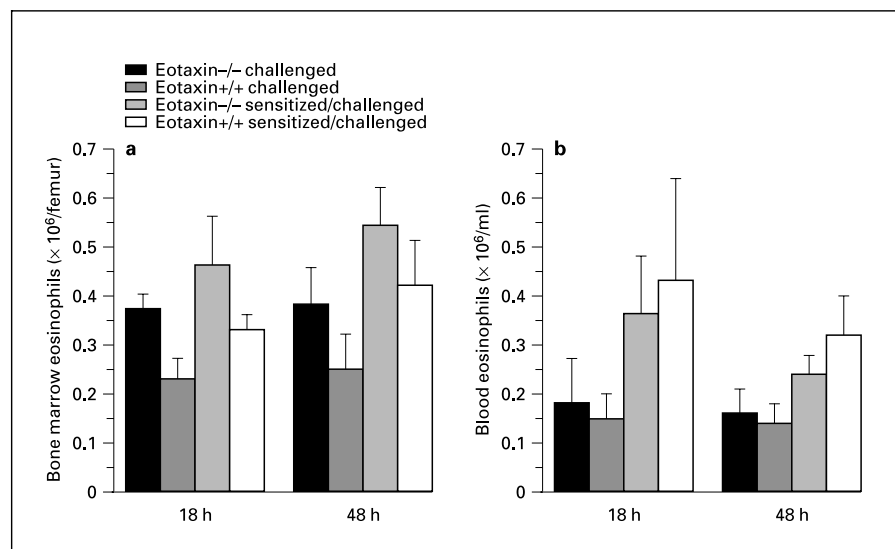
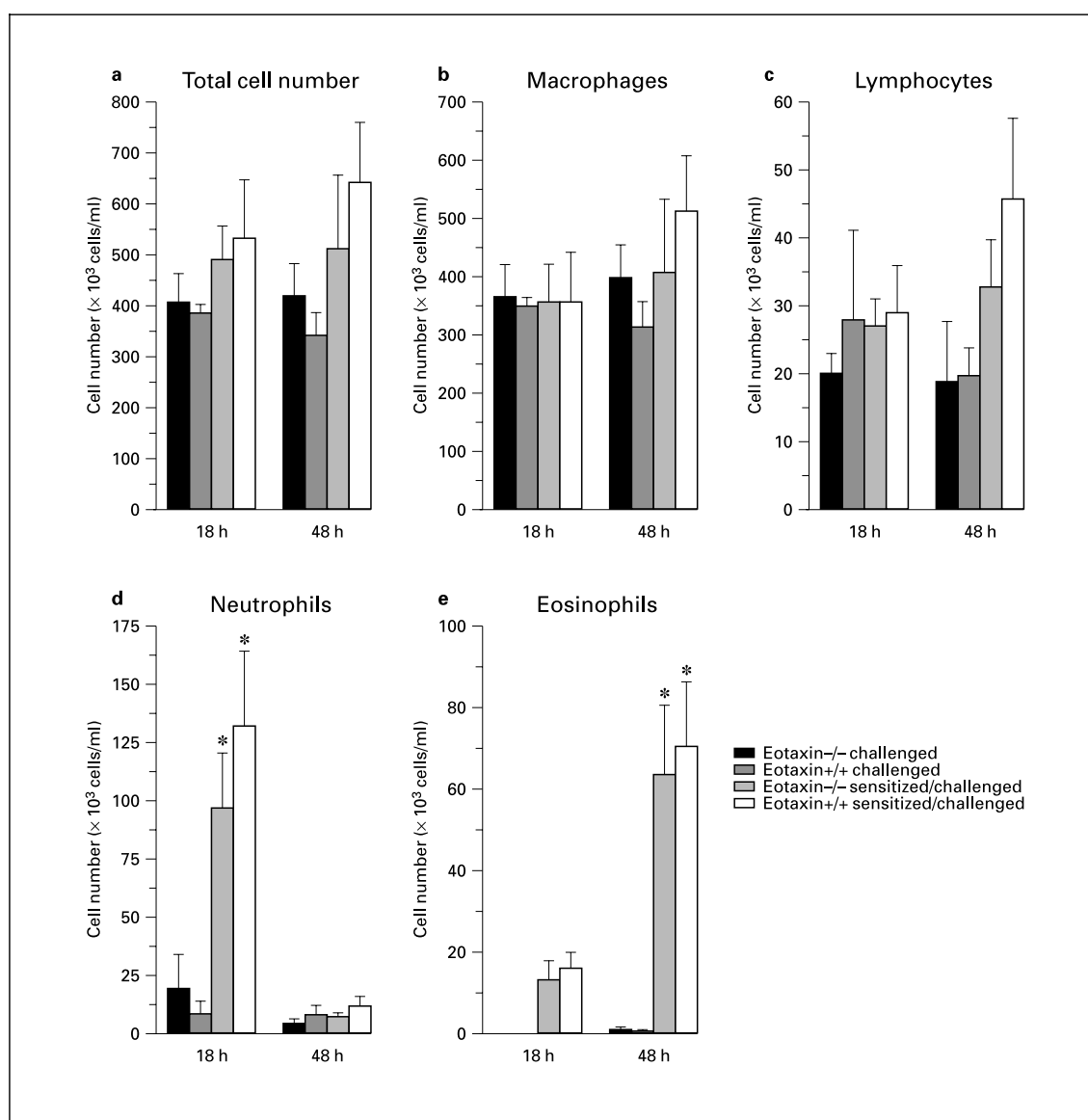


Fig. 2. Eotaxin-1^{-/-} mice develop normal bone marrow and blood eosinophilia following OVA sensitization and challenge. Eosinophil numbers were determined in the bone marrow (**a**) and blood (**b**) of eotaxin-1^{+/+} and eotaxin-1^{-/-} mice receiving OVA challenge alone (challenged), and in OVA-sensitized and challenged (sensitized/challenged) mice. Data represent the mean $\pm$ SEM (n = 5–7 per group). * Significant difference (p < 0.05) between challenged alone and sensitized and challenged groups.

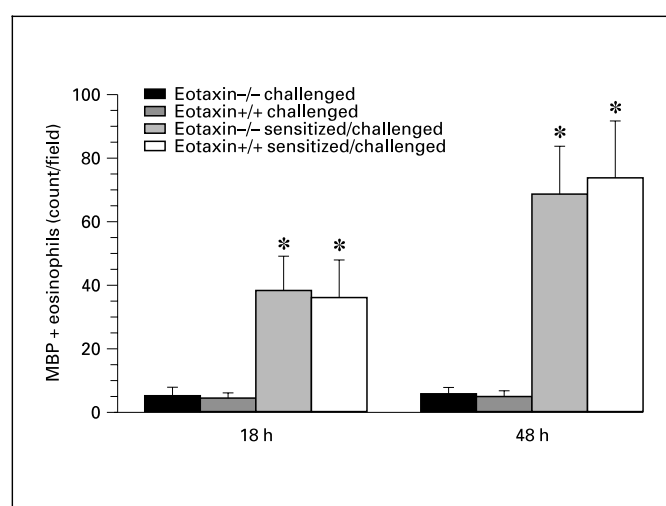




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Fig. 3. Eotaxin-1^{-/-} mice develop a significant BALF eosinophilia 48 h following OVA sensitization and challenge. Cellular composition of BALF was determined in eotaxin-1^{-/-} mice and eotaxin-1^{+/+} mice following OVA challenge alone (challenged), and in OVA-sensitized and challenged (sensitized/challenged) mice. Total cell number (a), macrophages (b), lymphocytes (c), neutrophils (d), eosinophils (e). Data represent the mean $\pm$ SEM (n = 5–7 per group). * Significant difference (p < 0.05) between challenged alone and sensitized and challenged groups.

Fig. 4. Eotaxin-1^{-/-} mice develop a significant tissue eosinophilia 18 and 48 h following OVA sensitization and challenge. Immunohistochemistry on lung tissue was performed using a MBP antibody in eotaxin-1^{-/-} mice and eotaxin-1^{+/+} mice following OVA challenge alone (challenged), and in OVA sensitized challenged (sensitized/challenged) mice. Eosinophil number was quantified (counts/field) as described in methods. Data represent the mean $\pm$ SEM (n = 5 per group). * Significant difference (p < 0.05) between challenged alone and sensitized and challenged groups.



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Discussion

Eotaxin has been identified as a potent chemoattractant for eosinophils, and as such has been implicated in the trafficking of eosinophils to the lung and the development of AHR in allergic asthmatics [12]. Previously, one study demonstrated a 70% reduction in eosinophil infiltration to the BALF 18 h after a single aerosol allergen challenge of OVA-sensitized, eotaxin-deficient mice [37]. However, at the later time point of 48 h, no difference in the number of infiltrating eosinophils in the BALF of eotaxin-deficient and eotaxin-sufficient mice was seen [37]. While this suggested that eotaxin may be important in the early recruitment of eosinophils to the lung, the effect on airway responsiveness was not assessed. In the present study the role of eotaxin in the development of allergic airway eosinophilia and AHR was investigated using eotaxin-1-deficient mice. Infiltration of eosinophilia into the BALF and airway tissue was evaluated at 18 and 48 h after a single aerosol challenge in OVA-sensitized eotaxin-1^{-/-} mice and eotaxin-1^{+/+} mice. Both groups of mice developed a low-level BALF eosinophilia at 18 h, which was further and similarly increased at 48 h. Moreover, both groups of mice developed a significant tissue eosinophilia at 18 h, which was correspondingly increased at 48 h. Although the difference in eosinophil recruitment seen in the present study could reflect a strain difference (129SvEv in the present study vs. Swiss Webster/129 mixed background), Yang et al. [38], using a different genetic background and different gene targeting strategy, were also unable to see any effect of eotaxin deficiency on the development of airway tissue eosinophilia at either 6, 12, or 24 h following a single aerosol challenge of OVA-sensitized mice.

In a model of cockroach allergen-induced airway eosinophilia, eotaxin neutralization was effective in reducing peribronchial eosinophil accumulation following secondary rechallenge of animals 48 h after a single intratracheal allergen challenge [31]. Interestingly, when a single intra-

tracheal allergen provocation was used, eotaxin antibody was ineffective in reducing eosinophil infiltration [31]. In contrast, neutralizing antibody to MIP-1 α was effective in reducing eosinophil accumulation after a single allergen challenge, but not the secondary rechallenge [31]. A second study evaluated the role of several chemokines in the recruitment of eosinophils to the lung during multiple allergen challenges of sensitized mice [36]. Antibody to eotaxin, administered throughout the challenge period, reduced BALF and tissue eosinophil accumulation. The effects of eotaxin on eosinophil infiltration, although reportedly transient, were more pronounced at the end of the multiple challenge period than at the start. A number of other chemokines, including RANTES, MIP-1 α , MCP-1 and MCP-5, were also implicated at different times in the recruitment of eosinophils to the lung. Thus, while eotaxin may play a role in the recruitment of eosinophils to the lung (and AHR to some extent), these and our own observations using eotaxin-deficient mice, suggest a complex network of chemokines may coordinate the eosinophil trafficking to the lung and that eotaxin-1 per se is not essential for allergic airway eosinophilic inflammation.

In summary, our observations suggest that eotaxin-1 is not critical for the development of airway eosinophilia and AHR in this single challenge model, implying that other chemokines or combinations of chemokines can compensate for the deficiency of eotaxin in eosinophilic recruitment to the airway during the allergic response.

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