

Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperreactivity

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Background: Eotaxin-2 is a member of the eotaxin subfamily of CC chemokines that display eosinophil-specific, chemotactic properties and has been associated with allergic disorders. However, the contribution of eotaxin-2 to the development of defined pathogenic features of allergic disease remains to be defined.

Objective: We sought to determine whether eotaxin-2 was a cofactor with IL-5 for the regulation of pulmonary eosinophilia and to identify the combined role of these molecules in the induction of phenotypic characteristics of allergic lung disease. **Methods:** We instilled recombinant eotaxin-2 into the airways of wild-type mice that had been treated systemically with IL-5 or into IL-5-transgenic mice and characterized pulmonary eosinophil numbers, IL-13 production, and airway hyperreactivity (AHR) to methacholine. Mice deficient in the IL-4 receptor α -chain, IL-13, and signal transducers and activators of transcription 6 or mice treated with anti-CCR3 monoclonal antibody were also used.

Results: Eotaxin-2 and IL-5 cooperatively promoted eosinophil accumulation, IL-13 production, and AHR to methacholine. Neither eotaxin-2 nor IL-5 alone induced these features of allergic disease. IL-13 production was critically dependent on eotaxin-2- and IL-5-regulated eosinophilia, which predisposed to the development of AHR. AHR was dependent on IL-13 and signaling through the IL-4R α -chain and signal transducers and activators of transcription 6 pathways and the presence of eosinophils in the lung.

Conclusion: These investigations demonstrate important cooperativity between eotaxin-2, IL-5, and IL-13 signaling systems and eosinophils for the development of hallmark features of allergic disease of the lung. (*J Allergy Clin Immunol* 2003;112:935-43.)

Key words: Airway hyperreactivity, eosinophils, eotaxin-2, interleukins-5 and -13, mouse model, cell recruitment

Blood and/or tissue eosinophilia is a hallmark feature of allergic disorders of the airways (rhinitis and asthma), skin (eczema), gastrointestinal tract (food allergy, parasitic infections, and allergic eosinophilic gastroenteritis), and numerous systemic diseases (eg, Churg-Strauss syndrome, eosinophil pneumonia, and the idiopathic hyper-eosinophilic syndrome).¹ Eosinophils are thought to play a pathologic role in these disease processes through the release of an array of cytotoxic products and proinflammatory mediators.^{1,2}

The proposed pathogenic role for eosinophils in allergic diseases has initiated extensive investigations into the molecular processes that selectively regulate eosinophil migration.¹ Although a wide range of molecules have been identified as eosinophil chemoattractants, only the eotaxin subfamily (eotaxins-1, -2, and -3) of CC chemokines are selective for eosinophil chemotaxis^{1,3,4} by activating CC chemokine receptor 3 (CCR3). Eotaxin-1 was first identified in bronchoalveolar lavage fluid (BALF) of the allergic airways of guinea pigs⁵ and has been shown to be an important factor in the regulation of baseline homing of eosinophils and a critical cofactor for IL-5-induced eosinophilia.⁶ Eotaxin-2 and eotaxin-3 were first described on the basis of sequence identity with eotaxin-1.⁷⁻¹⁰ Identification of the nucleotide sequences and chromosomal positioning of the eotaxin genes shows that eotaxins-1, -2, and -3 are distantly related. Eotaxins-2 and -3 have 30% to 40% sequence identity with eotaxin-1 and are located on human chromosome 7q11.23, whereas eotaxin-1 is positioned at 17q21.1.⁸⁻¹¹ In the mouse, only eotaxin-1 and eotaxin-2 have been identified.

Characterization of eotaxin-1, -2, and -3 expression, distribution, and biologic activity suggests that these 3 chemokines possess common biologic functions. All of the eotaxins are eosinophil chemoattractants, and although efficacy levels are very similar, eotaxin-1 is

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Abbreviations used

AHR:	Airway hyperreactivity
BALF:	Bronchoalveolar lavage fluid
CCR3:	Chemokine receptor 3
HPF:	High-powered field
IL-4R α :	IL-4 receptor α -chain
IT:	Intratracheal
IV:	Intravenous (injection)
mAb:	Monoclonal antibody
Penh:	Enhanced pause
STAT:	Signal transducers and activators of transcription
Tg:	Transgenic
WT:	Wild type

more potent than eotaxins-2 and -3 in inducing chemotaxis in vitro.^{8,9,12,13} Eotaxins-1 and -2 also induce rapid and transient actin polymerization, upregulate integrin function, and modulate respiratory burst in eosinophils.^{12,14} Both eotaxin-1 and -2 production is regulated by signal transducers and activator of transcription (STAT6).^{15,16} The potent effects of the eotaxins on eosinophil function are explained by their ability to signal exclusively through CCR3, which is primarily found on this granulocyte.¹⁷ Blockade of CCR3 by neutralizing monoclonal antibody (mAb) inhibits both eotaxin-1- and -2-induced Ca²⁺ flux in human eosinophils.¹² Furthermore, eotaxin-1 induces complete cross-desensitization of eotaxin-2 signaling, and both chemokines induce CCR3 internalization, confirming selective activation of CCR3.^{8,12,18,19}

Limited studies suggest that eotaxins-1, -2, and -3 play important roles in regulating different phases of eosinophil recruitment during allergic inflammation. Inhibition of eotaxin-1 activity by a neutralizing mAb or by gene disruption attenuates but does not abolish eosinophil accumulation in the lungs of allergic mice.²⁰⁻²³ Thus, other mechanisms contribute to eosinophil trafficking during allergic inflammation, and these might be linked to eotaxin-2 activity.¹³ Indeed, clinical studies suggest that there is differential utilization of eotaxins-1 and -2 in the recruitment of eosinophils to allergic tissue.²⁴ Eotaxins-1 and -2 also have differential patterns of expression, which might underpin defined biologic roles. Eotaxin-1 is constitutively expressed in the thymus and lung and throughout the length of the gastrointestinal tract (esophagus, stomach, and small intestine).^{13,21} By contrast, eotaxin-2 is primarily expressed in the spleen and jejunum.¹³ Interestingly, the expression of eotaxins-1 and -2 is upregulated in the airways of transgenic (Tg) mice overexpressing IL-4 in the lung and also after intranasal administration of recombinant IL-4.¹³ Furthermore, IL-4-induced expression of eotaxins-1 and -2 in the lung is dependent on STAT6 expression.¹³ IL-4 also stimulates expression of eotaxin-3 in human umbilical vein endothelial line cells, whereas IFN- γ upregulates eotaxin-1 production in these cells.

Collectively, the aforementioned studies suggest differential roles for the eotaxin subfamily of CC

chemokines in regulating various components of eosinophil trafficking and function during allergic inflammatory responses. However, unlike eotaxin-1, the roles of eotaxins-2 and -3 in regulating eosinophil migration, their relationship with IL-5 for promoting eosinophilia, and their involvement in the pathogenesis of allergic inflammatory responses have not yet been examined. In this investigation, we examined the role of eotaxin-2 as a cofactor for IL-5-induced eosinophil migration into the lung and in the induction of key pathogenic processes of allergic airway disease. We show important cooperative interplay between eotaxin-2 and IL-5 for the development of pulmonary eosinophilia, IL-13 production, and airway hyperreactivity (AHR).

MATERIALS AND METHODS

Mice

BALB/c wild-type (WT), eotaxin-1-deficient, and IL-5-Tg (F₁₄ generation BALB/c²⁵) mice (6 to 12 weeks old) were obtained from the specific pathogen-free facilities at the Australian National University, Canberra, Australia. IL-13²⁶ and STAT6²⁷ deficient mice were backcrossed 8 and 6 generations, respectively, with the BALB/c strain. BALB/c IL-4 receptor α -chain (IL-4R α)-deficient mice were a gift from Dr Nancy Noben-Trauth (National Institutes of Health, Bethesda, Md).²⁸ All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the Australian National University.

Induction of the allergic phenotype by eotaxin-2

Mice were anesthetized (intravenous [IV] injection of 100 μ L Saffan solution [1:4 diluted with PBS]) and then intubated with a 22-gauge catheter needle through which murine recombinant eotaxin-2 (Pepro Tech, Inc, Rocky Hill, NJ¹³; 3 μ g dissolved in 20 μ L vehicle: 0.1% BSA/PBS) or control vehicle (0.1% BSA/PBS) was instilled into the airways. AHR, mucus production, airway eosinophilia, and levels of IL-13 in the BALF were measured at 6 or 24 hours after instillation. In some experiments, murine recombinant IL-5 (50 ng dissolved in 200 μ L PBS) was administered IV on 4 consecutive days (at 24-hour intervals) to WT mice followed by intratracheal (IT) instillation of IL-5 (1 μ g) and/or eotaxin-2 (3 μ g) (dissolved in a final volume of 20 μ L vehicle), and airway responses were analyzed 6 or 24 hours later. Mouse recombinant IL-5 was expressed and purified to homogeneity as previously described.²⁹

Inhibition of the effects of eotaxin-2 by IV administration of eotaxin-1

IL-5-Tg mice were injected IV with eotaxin-1 (0.6 μ g/200 μ L vehicle: 0.1% BSA/PBS per mouse; Pepro Tech, Inc, Rocky Hill, NJ), and 30 minutes later eotaxin-2 (3 μ g) or vehicle control was instilled into the airways as described earlier. AHR, eosinophilia, and levels of IL-13 in BALF were measured 24 hours after eotaxin-2 treatment.

Measurement of airway reactivity to methacholine

Airway reactivity to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography (Buxco, Troy, NY) as previously described.³⁰ In brief, mice were placed in the plethysmograph chamber, and baseline readings were averaged for 3 minutes. Aerosolized methacholine (doubling concentrations in solution ranging from 3.125 to 50 mg/mL) was then delivered through an

inlet into the chamber for 2 minutes, and readings were averaged over another period of 3 minutes after each dose was administered. Data presented represent the percent increase in enhanced pause (Penh) at 25 mg/mL methacholine over baseline reactivity in the absence of cholinergic stimuli. This concentration of methacholine induced maximal responsiveness, and the data are reflective of the full dose-response curve obtained to methacholine.

Measurement of IL-13 in BALF

The concentration of IL-13 from cell-free BALF supernatant was determined by ELISA using the antibodies AB-413-NA and AF-413-NA (R&D Systems, Minneapolis, Minn) as previously described.³⁰

Characterization of eosinophils in lung tissue

Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with Carbol's chromotrope-hematoxylin for identification of eosinophils. The numbers of eosinophils in the central bronchi-bronchiole area were identified by morphological criteria and quantified as previously described.^{30,31} Data are presented as the number of peribronchial/perivascular eosinophils per 10 high-powered fields (HPFs; $\times 40$ magnification).

Treatment of mice with anti-CCR3 mAb

Mice were injected intraperitoneally with anti-mouse CCR3 (200 μ L of 1 mg mAb in saline; 6S2-19-4; DNAX, Palo Alto, Calif) or control mAb (200 μ L of 1 mg control mAb [IgG isotype match] in saline) 1 day before the first IV administration of IL-5 in naive WT mice. IL-5 (50 ng dissolved in 200 μ L PBS) was then administered IV daily for 4 consecutive days to naive WT mice, followed by IT instillation of IL-5 (1 μ g) and eotaxin-2 (3 μ g; dissolved in a final volume of 20 μ L vehicle) on day 4, and airways were analyzed 24 hours later.

Statistical analysis

The significance of differences between means of experimental groups was analyzed using Student's unpaired *t* test. Data are reported as the mean $\pm$ SEM. Differences in mean values were considered significant when *P* < .05.

RESULTS

Effect of eotaxin-2 on pulmonary eosinophil numbers and AHR in WT and IL-5-Tg mice

IT instillation of 3 μ g of eotaxin-2 did not induce AHR, mucus secretion, or peripheral blood, BALF, or tissue eosinophilia in WT mice (results not shown). By contrast, IT instillation of 3 μ g of eotaxin-2 to IL-5-Tg mice decreased peripheral blood eosinophil numbers (results not shown), which were correlated with a significant increase in BALF eosinophils (vehicle, $2.0 \pm 1.8 \times 10^3$ eosinophils/mL vs eotaxin-2, $2.35 \pm 0.95 \times 10^5$ eosinophils/mL; mean of *n* = 6 per group, *P* < .05) and tissue eosinophil numbers (Fig 1, A). The recruitment of eosinophils into pulmonary tissues of IL-5-Tg mice by eotaxin-2 directly correlated with the induction of AHR (Fig 1, B). By contrast with WT mice, eotaxin-2 also promoted mucus secretion in IL-5-Tg mice (vehicle, 6.18 ± 2.22 mucus-staining cells/HPF vs eotaxin-2, 25.29 ± 0.99 mucus-staining cells/HPF; mean of *n* = 6 per group, *P* < .05]. These features of allergic airway disease, induced by

eotaxin-2 in IL-5-Tg mice, were dose dependent and maximal at 3 μ g. Differential analysis of cell populations in the BALF indicated that eotaxin-2 (3 μ g) selectively recruited eosinophils to the airways as lymphocyte, neutrophil, and macrophage numbers were not altered when compared with vehicle treatment (results not shown).

Role of IL-5 and eotaxin-1 in the onset of eotaxin-2-induced pulmonary eosinophilia and AHR

To further investigate the interplay between IL-5, eosinophils, and eotaxin-2 in the induction of AHR, we developed an experimental model of eotaxin-2-induced airway eosinophilia and AHR in WT mice (Fig 1, C and D). WT mice were administered IL-5 IV over 4 days to promote peripheral eosinophil migration,³⁰ and they received IL-5 and eotaxin-2 IT to promote pulmonary eosinophilia. Administration of IL-5 to the airways in the presence of IV IL-5 did not promote eosinophil accumulation in the lung or AHR (Fig 1, C and D). Administration of eotaxin-2 to the lung in the presence of IV IL-5 slightly increased the number of eosinophils in tissues but did not promote AHR (data not shown). However, IT administration of both IL-5 and eotaxin-2 in the presence of IV IL-5 promoted pulmonary eosinophilia concurrently with the development of AHR (Fig 1, C and D). Collectively, these findings suggest that eotaxin-2-induced pulmonary eosinophilia and AHR are dependent on the presence of eosinophils and elevated pulmonary IL-5 levels. The observation that IT instillation of IL-5 after IV IL-5 treatment did not induce a pulmonary eosinophilia or AHR suggests that IL-5 in the pulmonary compartment alone is not sufficient for the induction of these responses.

These findings were supported by results obtained using IT instillation of eotaxin-2 to IL-5-Tg mice (Fig 1, A and B). At baseline, the level of pulmonary eosinophilia in IL-5-Tg mice is ~ 10 -fold higher than that observed in eotaxin-2/IL-5-IT- and IL-5-IV-treated WT mice (Fig 1, A and C, respectively). However, there was no significant difference in the level of airway reactivity at baseline when compared with vehicle-treated WT or IL-5-Tg mice (Fig 1, D and B, respectively). IT instillation of eotaxin-2 significantly increased the pulmonary eosinophil numbers in IL-5-Tg mice compared with vehicle-treated IL-5-Tg mice (Fig 1, A), and this increase was directly associated with the induction of AHR (Fig 1, B). The interplay between eotaxin-2- and IL-5-regulated eosinophilia is therefore critical for the induction of AHR.

We also determined the role of eotaxin-1 in eotaxin-2-induced responses. IT administration of both eotaxin-2 and IL-5 in the presence of IV IL-5 promoted pulmonary eosinophilia (eosinophils/HPF: eotaxin-1 deficient, 2.11 ± 0.3 ; WT, 1.59 ± 0.6) concurrently with the development of AHR (percent increase in Penh over baseline at 25 mg methacholine: eotaxin-1 deficient, 711.3 ± 138.2 ; WT, 890 ± 107.4) in eotaxin-1-deficient mice, and these responses were not significantly different (*P* > .05, *n* = 4 to 6 mice per group) than those obtained in WT mice.

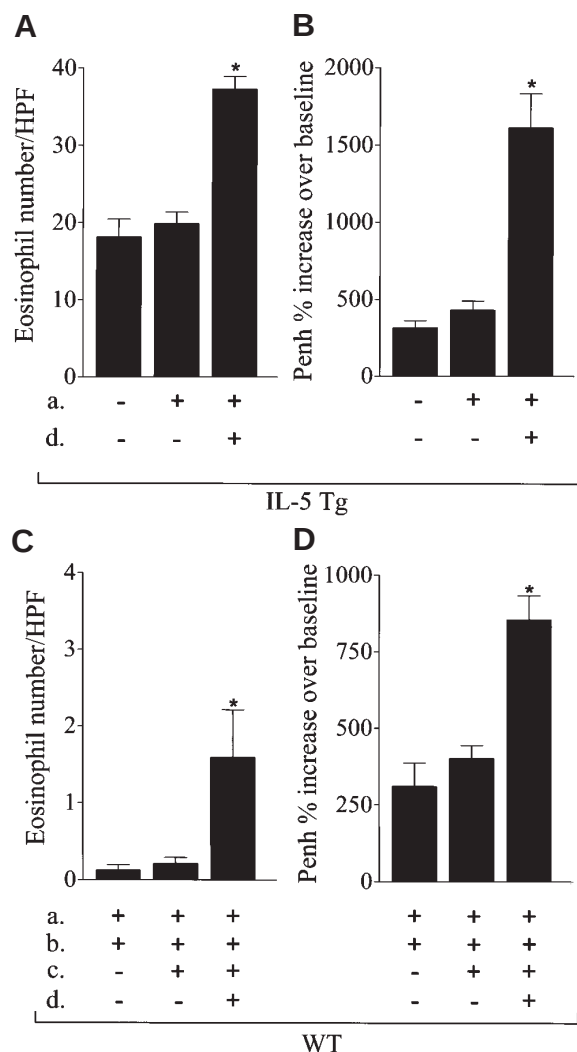


FIG 1. The effect of eotaxin-2 on the levels of eosinophils in the lung and airway reactivity in WT and IL-5-Tg mice. Effect of eotaxin-2 on (A) eosinophil numbers in lung tissue and (B) AHR in IL-5-Tg mice. Effect of eotaxin-2 on (C) eosinophil numbers in lung tissue and (D) AHR in WT mice. a, vehicle (IT); b, IL-5 (4×50 ng IV); c, IL-5 ($1 \mu\text{g}$ IT); d, eotaxin-2 ($3 \mu\text{g}$ IT). Data are mean $\pm$ SEM from $n = 4$ to 6 mice per group. * $P < .05$ compared with other groups. Responses obtained from vehicle treatment were not significantly different from nontreated or vehicle-treated plus IL-5 IV groups (data not shown).

Role of eosinophils in the induction of eotaxin-2-induced AHR

To identify the direct contribution of eosinophils to the induction of eotaxin-2-mediated AHR, IL-5 was administered IV to WT mice treated with anti-CCR3 mAb, an eosinophil-depleting antibody, followed by IT challenge with IL-5 and eotaxin-2. Treatment of mice with anti-CCR3 mAb abolished eotaxin-2-induced AHR and the recruitment of eosinophils to the blood (result not shown) and lung (Fig 2, A and B).

Eotaxin-2 promotes the upregulation of pulmonary IL-13 levels and mucus secretion in IL-5-Tg mice

To further clarify the mechanism whereby eotaxin-2 induced AHR, we determined IL-13 levels in BALF after eotaxin-2 (IT) and IL-5 (IT and IV) challenge of WT mice and the delivery of eotaxin-2 (IT) to IL-5-Tg mice (Fig 3, A through C). IT instillation of eotaxin-2 to IL-5-Tg mice or WT mice treated with IL-5 induced a significant increase in BALF IL-13 levels compared with vehicle-treated mice (Fig 3, A and B).

To identify whether eotaxin-2-induced upregulation of IL-13 was dependent on eosinophils, WT mice were challenged with IL-5 (IV and IT) in the presence or absence of eotaxin-2 (Fig 3, B). No significant increase in IL-13 levels was observed after IV and/or IT administration of IL-5 in the absence of eotaxin-2 (Fig 2, B). These data suggest that elevated levels of IL-5 in the blood and lung are insufficient to promote IL-13 production in respiratory secretions. Only after both IV and IT administration of IL-5 and IT instillation of eotaxin-2 was a significant increase in BALF IL-13 levels observed (Fig 3, B). Treatment of WT mice exposed to eotaxin-2 and IL-5 (IV and IT) with anti-CCR3 mAb inhibited the production of IL-13 (Fig 3, C). Collectively, the aforementioned data (Figs 2 and 3) suggest that eosinophils play an important role in IL-13 production in the lung in response to eotaxin-2 treatment.

We also treated IL-5-Tg mice IV with eotaxin-1 before IT administration of eotaxin-2. Eotaxin-1 pretreatment significantly inhibited the recruitment of eosinophils to the lung (cells/mL BALF: eotaxin-2 IT plus eotaxin-1 IV, $1.64 \pm 0.65 \times 10^5$; eotaxin-2 IT plus vehicle IV, $10.5 \pm 0.2 \times 10^5$), production of IL-13 (IL-13 ng/mL BALF: eotaxin-2 IT plus eotaxin-1 IV, 0.33 ± 0.02 ; eotaxin-2 IT plus vehicle, 2.0 ± 0.41), and the development of AHR (percent increase in Penh over baseline at 25 mg methacholine: eotaxin-2 IT plus eotaxin-1 IV, 365.6 ± 87.0 ; vs eotaxin-2 IT plus vehicle IT, 1611 ± 220 ; $P < .01$, $n = 4$ to 6 mice per group; vehicle IT, 429 ± 60). Because eotaxin-1 specially binds to CCR3, these data show that eotaxin-2 directly regulates eosinophil migration through CCR3 and that induction of IL-13 production and AHR is dependent on the recruitment of this cell type to the lung.

Eosinophilia precedes IL-13 production in the lung

To determine the relationship between IL-13 production and the accumulation of eosinophils in the lung, we delivered eotaxin-2 IT to IL-5-Tg mice and measured responses at 6 hours. Although eosinophil numbers were significantly elevated at this time (cells/mL BALF: eotaxin-2, $8.8 \pm 1.5 \times 10^5$; vehicle, < 100), the levels of IL-13 and airway reactivity were similar to those in untreated mice (data not shown). By contrast, IL-13 levels and airway reactivity were significantly elevated at 24 hours, and these processes were dependent on eosinophil

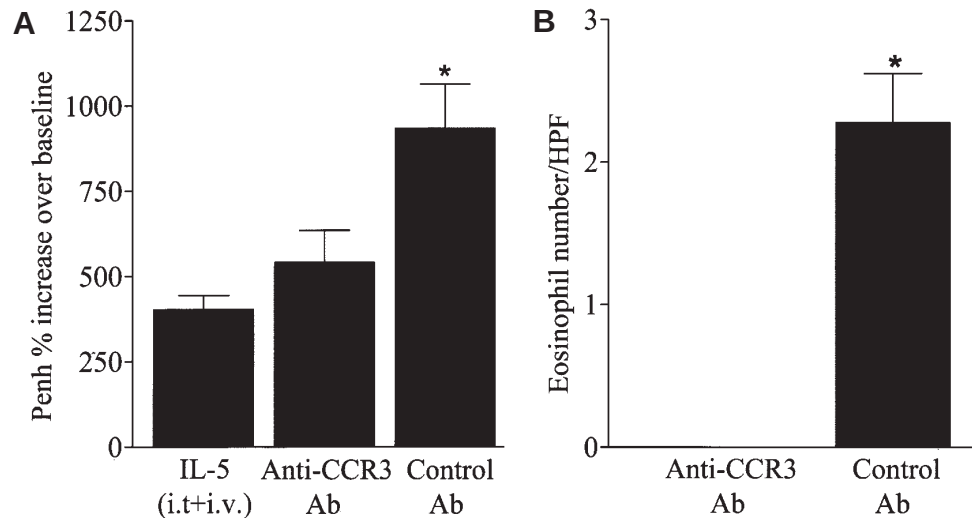


FIG 2. The effect of anti-CCR3 mAb on eotaxin-2-induced AHR and eosinophil numbers in the lung tissue of WT mice. Effect of anti-CCR3 mAb treatment on eotaxin-2 induced (A) AHR and (B) eosinophil accumulation in the lung. The degree of eosinophilia and AHR in control mAb-treated mice was not significantly different from that observed in its absence (Fig 1, C and D). The level of AHR in anti-CCR3-mAb-treated mice was not significantly different from IL-5 (IV and IT) treatment or baseline responsiveness (Fig 1, D). Data are mean $\pm$ SEM from $n = 4$ to 6 mice per group. * $P < .05$ compared with anti-CCR3 mAb group.

accumulation in the lung (Figs 1 through 3). To determine the potential cellular source of IL-13 at 24 hours, we isolated eosinophils, lymphocytes, and macrophages from the BALF by flow cytometry and performed reverse transcription-PCR for IL-13. Eosinophils were the predominant cellular infiltrate in the BALF at this time and the primary source of IL-13 expression (data not shown).

Role of the IL-4R α -chain STAT6, and IL-13 in the induction of eotaxin-2-mediated pulmonary eosinophilia, AHR, and production of IL-13

To identify the contribution of IL-4R α -chain IL-13, and STAT6 in the induction of eotaxin-2-mediated eosinophil infiltration and/or AHR, IL-5 was administered IV to mice deficient in these factors, followed by IT challenge with IL-5 and eotaxin-2. Eotaxin-2-mediated AHR was critically dependent on the IL-4R α -chain, IL-13, and STAT6 (Fig 4, A). Eotaxin-2-induced pulmonary eosinophilia was not dependent on IL-4R α -chain, IL-13, or STAT6 expression (Fig 4, B). The lack of IL-5- and eotaxin-2-inducible AHR in IL-4R α -chain-, IL-13-, and STAT6-deficient mice directly correlated with the inability of these mice to produce significant levels of IL-13 in the lung in response to eotaxin-2 administration (Fig 4, C).

Eotaxin-2 induces pulmonary eosinophilia and IL-13 production but not mucus hypersecretion or AHR in IL-5-Tg/STAT6-deficient mice

To further elucidate the contribution of IL-5 and eosinophils to the induction of eotaxin-2-induced AHR

in the lung, we generated IL-5-Tg mice deficient in STAT6. By comparison with IL-5-Tg mice, delivery of eotaxin-2 to the airways did not induce mucus hypersecretion (results not shown) or AHR in IL-5-Tg/STAT6-deficient mice (Fig 5, A and B). However, eotaxin-2 induced the recruitment of eosinophils to the airway tissue and promoted the secretion of IL-13 into BALF (Fig 5, C). These data support the results in WT mice (Figs 2 and 3) showing that eosinophils recruited to the airways play an important role in the production of IL-13 in the lung and that this cytokine subsequently promotes AHR by STAT6-dependent pathways.

DISCUSSION

Although the CC chemokines eotaxins-1, -2, and -3 are known to selectively regulate eosinophil migration, their individual contribution (in particular, eotaxins-2 and -3) to the induction of pathologic features of allergic disease and the mechanism(s) whereby these molecules might regulate disease remain obscure. In the present study, we show that eotaxin-2 is an important cofactor with IL-5 for the selective recruitment of eosinophils into the airways. Moreover, IL-5- and eotaxin-2-regulated eosinophilia predisposes to the production of IL-13 in the lung, and this cytokine induces AHR. Thus, we show that integration of signals elicited by IL-5 and eotaxin-2 play fundamental roles in the regulation of key pathogenic processes of allergic disease.

Delivery of eotaxin-2 to the lung resulted in pulmonary eosinophilia and AHR in IL-5-Tg mice but not in WT mice. However, eosinophil accumulation in pulmonary tissues and AHR in response to eotaxin-2 were observed in WT mice after administration of IL-5 to the

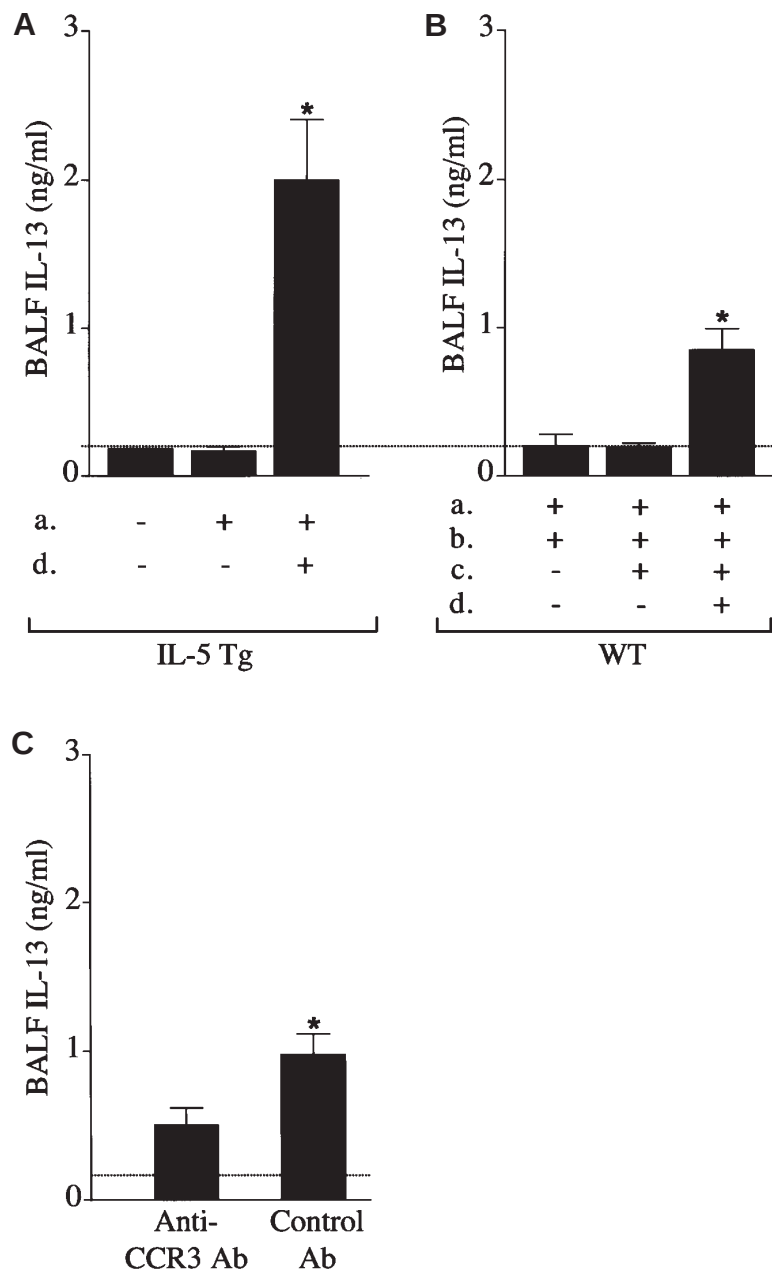


FIG 3. The effect of eotaxin-2 on IL-13 levels in the BALF of WT and IL-5-Tg mice. The effect of eotaxin-2 on BALF IL-13 levels from (A) IL-5-Tg mice, (B) WT mice treated with IL-5, and (C) WT mice treated with IL-5 (IV and IT as in B) and anti-CCR3 mAb or control antibody. a, Vehicle (IT); b, IL-5 (4×50 ng IV); c, IL-5 (1μ g IT); d, eotaxin-2 (3μ g IT); broken line, level of detection. Data represent mean $\pm$ SEM from $n = 4$ to 6 mice per group. A and B, $*P < .05$ compared with vehicle or nontreatment group; C, $*P < .05$ compared with anti-CCR3 mAb group. The level of IL-13 in the anti-CCR3 mAb group was not significantly different from background levels.

lung and blood. These data indicated that eotaxin-2-induced pulmonary eosinophilia was critically dependent on the presence of IL-5. These findings were further supported by the observation that eotaxin-2 could not recruit eosinophils into the pulmonary compartment or promote IL-13 production in mice treated with anti-CCR3 mAb, where eosinophils were absent in the circulation. We have previously demonstrated a central role for IL-5 in

the induction of blood eosinophilia and the recruitment of eosinophils into pulmonary tissue.^{31,32} We have also shown a unique cooperative interplay between eotaxin-1 and IL-5 for the regulation of tissue eosinophilia.^{25,32} It would appear that eotaxin-2, like eotaxin-1, participates in a mechanism that selectively amplifies IL-5-induced responses. Notably, although both chemokines (eotaxins-1 and -2) signal through CCR3, they appear to exert indi-

vidual effects by regulating eosinophilia during different phases of the allergic response.²⁴ Indeed, in this model of eosinophil trafficking, eotaxin-1 was not required for the induction of eotaxin-2-regulated processes.

IL-5-regulated eosinophilia has been directly associated with the induction of AHR in mouse models of asthma.³¹ Although the mechanism by which AHR is induced remains obscure, recently it has been linked to eosinophil-regulated IL-13 production in the pulmonary compartment.²⁵ In this investigation, we show that eotaxin-2, in association with IL-5, promotes tissue eosinophilia that subsequently results in the induction of AHR. Moreover, eotaxin-2 was shown to stimulate, in an IL-5- and eosinophil-dependent manner, the upregulation of IL-13 levels in respiratory secretions. The ability of eotaxin-2 to promote IL-13 production and AHR appeared to be directly related to its ability to promote eosinophilia by signaling through CCR3. Anti-CCR3 mAb completely blocked eotaxin-2-induced recruitment of eosinophils into the lung and the induction of IL-13 production and AHR. IV delivery of eotaxin-1 also blocked the effects of eotaxin-2, further indicating that this chemokine regulates IL-13 production and AHR by directly acting on eosinophils. Exposure of eosinophils to eotaxin-1 induces internalization of CCR3,¹⁸ and we have recently shown that this process occurs in the circulation when eosinophils are exposed to IV eotaxin-1 (authors' unpublished data), thus inhibiting the ability of eotaxin-2 to signal and recruit this leukocyte from the blood to the lung. Temporal analysis of eotaxin-2-induced responses showed that eosinophil accumulation in the lung (6 hours after eotaxin-2 exposure) preceded the production of IL-13 and the induction of AHR. However, the subsequent induction of IL-13 production and AHR was dependent on the recruitment of eosinophils into the airways. Notably, elevated levels of IL-13 in the BALF at 24 hours directly correlated with the expression of this cytokine in eosinophils.

Even though IL-5 and eotaxin-2 treatment could induce tissue eosinophilia in the lungs of IL-4R α -, IL-13-, and STAT6-deficient mice, AHR was not observed in any of these animals. None of these strains produced significant quantities of IL-13 in response to combined treatment with IL-5 and eotaxin-2. Abolition of eosinophils in pulmonary tissues by anti-CCR3 mAb treatment also inhibited the production of IL-13 by eotaxin-2. Collectively, these data suggest a key role for IL-4R α -chain, STAT6, and eosinophils in the production of IL-13 and that this cytokine subsequently regulates the development of AHR through the IL-4R α -chain/STAT6 pathway. This interpretation is further supported by our experiments with IL-5-Tg mice deficient in STAT6. In these mice, an overabundance of IL-5 and eosinophils is associated with increased baseline and eotaxin-2-induced production of IL-13, but AHR and mucus hypersecretion do not occur. These experiments show that eosinophilia and IL-13 production precede the development of these responses through STAT6-dependent pathways. Analysis of IL-13 production in BALF cells at 24 hours strongly suggests

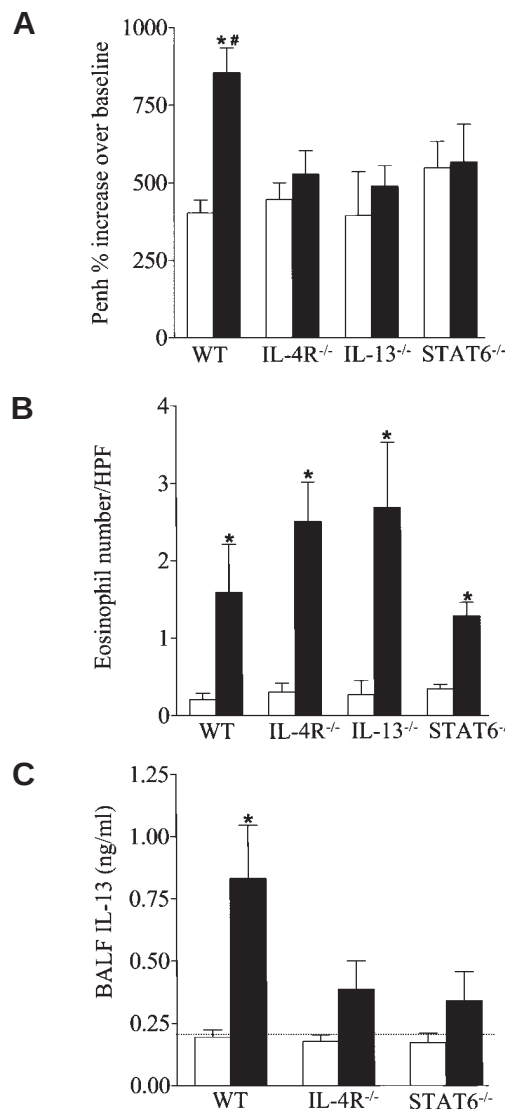


FIG 4. The effect of eotaxin-2 on the development of AHR, eosinophilia, and IL-13 production in the lung of WT and IL-4R α -chain-, IL-13-, and STAT6-deficient mice. Open bar, IL-5 (IV + IT); filled bar, eotaxin-2 (IT) + IL-5 (IV + IT); broken line, level of detection. The effect of eotaxin-2 on airways reactivity (**A**), eosinophil numbers (**B**), and IL-13 production (**C**) in WT and IL-4R α -, IL-13-, and STAT6-deficient mice. * $P < .05$ compared with IL-5 treatment only (IT + IV IL-5); # $P < .05$ compared with all treatments of IL-4R α -, IL-13-, and STAT6-deficient mice.

that the source of eotaxin-2- and IL-5-induced IL-13 can largely be attributed to the infiltrating eosinophil.

In the present study, we show that eotaxin-2 acts as an important cofactor with IL-5 in regulating pulmonary eosinophilia. We propose that once recruited to the lung, eosinophils become an important cellular source of IL-13, which subsequently can induce AHR through the IL-4R α -chain and STAT6. These findings establish the potential importance of eotaxin-2 in the induction of key phenotypic characteristics of experimental asthma and assist in clarifying the link between eosinophils and IL-13 for the induction of AHR.

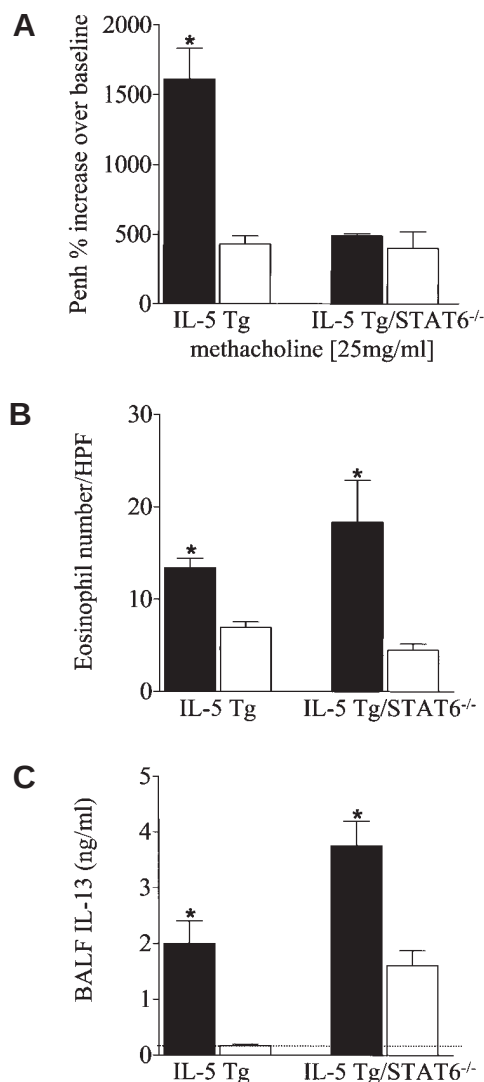


FIG 5. Eotaxin-2 induces pulmonary eosinophilia and IL-13 production but not AHR in IL-5-Tg/STAT6-deficient mice. Open bar, vehicle (IT); filled bar, eotaxin-2 (IT); broken line, level of detection. The effect of eotaxin-2 on airway reactivity (**A**), eosinophil numbers (**B**), and IL-13 levels (**C**) in IL-5-Tg/STAT6-deficient mice and IL-5-Tg mice. Data are mean $\pm$ SEM from $n = 4$ to 6 mice per group. * $P < .05$ compared with corresponding control-treated group.

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