

# IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism

Samuel M. Pope, BA,<sup>a,b</sup> Eric B. Brandt, PhD,<sup>a</sup> Anil Mishra, PhD,<sup>a</sup> Simon P. Hogan, PhD,<sup>a,c</sup> Nives Zimmermann, MD,<sup>a</sup> Klaus I. Matthaei, PhD,<sup>c</sup> Paul S. Foster, PhD,<sup>c</sup> and Marc E. Rothenberg, MD, PhD<sup>a</sup> Cincinnati, Ohio, and Canberra, Australia

**Background:** IL-13 induces several characteristic features of asthma, including airway eosinophilia, airway hyperresponsiveness, and mucus overproduction; however, the mechanisms involved are largely unknown.

**Objective:** We hypothesized that IL-13-induced inflammatory changes in the lung were dependent in part on IL-5 and eotaxin, two eosinophil-selective cytokines.

**Methods:** Recombinant murine IL-13 was repeatedly administered to the lung by intranasal delivery until the characteristic features of asthma developed. To analyze the role of IL-5 and eotaxin, we subjected eotaxin gene-targeted, IL-5 gene-targeted, eotaxin/IL-5-double-deficient, IL-5 transgenic, and wild-type mice of the Balb/C background to the experimental regime.

**Results:** The induction of IL-13-mediated airway eosinophilia was found to occur independently of eosinophilia in the blood or bone marrow, indicating that IL-13-induced airway inflammation is primarily mediated by local effects of IL-13 in the lung. Eosinophil recruitment into both the lung tissue and bronchoalveolar lavage fluid was markedly attenuated in IL-5-deficient mice in comparison with wild-type controls. Accordingly, IL-13 delivery to IL-5 transgenic mice resulted in a large increase in airway eosinophils in comparison with wild-type mice. Interestingly, IL-13-induced eosinophilia in the bronchoalveolar lavage fluid of eotaxin-deficient mice was not impaired; however, these same mice failed to mount a significant tissue eosinophilia in response to IL-13. Finally, IL-13-induced mucus production was not affected by the presence of IL-5 or eotaxin, suggesting that IL-13-induced mucus secretion is mechanistically dissociated from airway eosinophilia.

**Conclusion:** Selective components of the IL-13-induced asthma phenotype—airway eosinophilia but not mucus secretion—are differentially regulated by IL-5 and eotaxin. IL-5 is

required for IL-13 to induce eosinophilia throughout the lung, whereas eotaxin regulates the distribution of airway eosinophils. (*J Allergy Clin Immunol* 2001;108:594-601.)

**Key words:** Asthma, allergy, interleukin, chemokine, eotaxin

Eosinophil recruitment into inflammatory sites is a complex process regulated by a number of cytokines, including IL-3, GM-CSF, IL-6, IL-9, chemokines (eg, RANTES and eotaxin), and T<sub>H</sub>2 cell products (eg, IL-4, IL-5, and IL-13).<sup>1-3</sup> IL-13 appears to be particularly important, because it is produced in high quantities by T<sub>H</sub>2 cells and regulates multiple features of asthma (IgE production, mucus overproduction, eosinophil recruitment and survival, airway hyperreactivity, and the expression of CD23, adhesion molecules, and chemokines [eg, eotaxin]).<sup>4</sup> Dysregulation of IL-13 has been reported in a variety of allergic conditions, including asthma,<sup>5</sup> atopic dermatitis,<sup>6-8</sup> and allergic rhinitis.<sup>9-11</sup> Overexpression of IL-13 by pharmacologic administration or transgenic approaches induces multiple features of asthma, including eosinophilia, mucus overproduction, and airway hyperresponsiveness.<sup>12,13</sup> Importantly, neutralization of IL-13 activity by treatment with the soluble form of the IL-13 receptor  $\alpha$  2 chain (IL-13R $\alpha_2$ ) reverses many of the characteristic features of experimental asthma.<sup>14,15</sup>

Of the cytokines implicated in modulating leukocyte recruitment in response to IL-13, only IL-5 and eotaxin selectively regulate eosinophil trafficking.<sup>16</sup> IL-5 regulates growth, differentiation, activation, and survival of eosinophils and has been shown to provide an essential signal for the expansion and mobilization of eosinophils from the bone marrow into the lung after allergen exposure.<sup>17,18</sup> Eotaxin, an eosinophil-selective CC chemokine produced primarily by respiratory epithelial cells in the lungs, is induced by allergen challenge in experimental asthma in human beings and rodents and has been implicated in regulating eosinophil tissue recruitment.<sup>16,19</sup> Through the use of experimental models of antigen-induced airway inflammation, eotaxin has been demonstrated to partially regulate eosinophil recruitment during the late-phase response after repetitive mucosal antigen challenges.<sup>20,21</sup> Evidence has mounted indicating that eotaxin cooperates with IL-5 to regulate antigen-driven airway eosinophil recruitment.<sup>17,18,22,23</sup> In particular, IL-5 primes eosinophils to respond to eotaxin *in vitro*, and eotaxin's activity *in vivo* is enhanced by the presence of elevated levels of IL-5. Taken together, these data support

From <sup>a</sup>the Division of Pulmonary Medicine, Allergy and Clinical Immunology, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati; <sup>b</sup>the Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine; and <sup>c</sup>the Division of Biochemistry and Molecular Biology, The John Curtin School of Medical Research, Australian National University.

Supported in part by the National Health Medical Research Council (Australia) C.J. Martin Post-doctoral Fellowship (S.P.H.), NIH grants R01 AI42242-03 (M.E.R.) and R01 AI45898-02 (M.E.R.), the Human Frontier Science Program (M.E.R., P.S.F.), and the Postdoctoral Fellowship Award from the American Heart Association (E.B.B.).

Received for publication May 18, 2001; revised June 28, 2001; accepted for publication July 11, 2001.

Reprint requests: Marc E. Rothenberg, MD, PhD, Division of Pulmonary Medicine, Allergy and Clinical Immunology, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati, OH 45229.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/83/118600

doi:10.1067/mai.2001.118600

*Abbreviations used*

BALF: Bronchoalveolar lavage fluid  
MBP: Major basic protein

an essential role for IL-5 and eotaxin in regulating antigen-driven eosinophil recruitment into the lung.

The mechanism by which IL-13 induces airway eosinophilia has not been determined. It has been proposed that the effect of IL-13 might be chemokine-dependent, inasmuch as IL-13 is a potent inducer of eosinophil-active chemokines, including eotaxin and RANTES.<sup>13</sup> However, IL-13 also directly stimulates eosinophil activation, chemoattraction, and survival *in vitro*.<sup>24-26</sup> These findings suggest that IL-13 might be capable of inducing airway eosinophilia independently of IL-5. However, the specific role of individual chemokines and IL-5 has not been examined. To provide insight into the mechanisms that regulate the recruitment of eosinophils into the lung in association with IL-13 overexpression in asthma, we have established an experimental model that uses IL-13 to induce features of asthma. This model allows us to test the hypothesis that IL-13-induced responses in the airway (eg, eosinophilia and mucus production) are dependent on the eosinophil-specific cytokines IL-5 and eotaxin. Our data establish that exposure to intranasal IL-13 promotes eosinophil infiltration into the lung by a mechanism that is coregulated by IL-5 and eotaxin and dissociates mucus production from eosinophil recruitment.

## MATERIALS AND METHODS

### Mice

Eight- to 12-week-old male and female IL-5 gene-targeted, IL-5 transgenic, eotaxin-deficient, eotaxin/IL-5-double-deficient, and wild-type mice (Taconic Farms, Germantown, Pa) of the Balb/c background were generated and maintained under specific pathogen-free conditions, as described previously.<sup>20,27</sup>

### IL-13-induced airway responses

A mouse model of allergic lung disease was established by modifying previously described protocols.<sup>28,29</sup> Mice were lightly anesthetized by metofane inhalation (methoxy-fluorane; Pittman-Moore, Mundelein, Ill) and held in the supine position; 50  $\mu$ L normal saline solution or 10  $\mu$ g of recombinant murine IL-13 (generated in an HEK cell line [a generous gift of Dr Debra Donaldson, Genetics Institute, Cambridge, Mass]) was applied to the nares of each animal through use of a micropipette. After instillation, the mouse was held upright until alert. We have determined that with this procedure, approximately 70% of the solution is aspirated into the lung and 30% is administered to the gastrointestinal tract.<sup>30</sup> Mice were killed from 16 to 20 hours after the daily intranasal challenges.

### Bronchoalveolar lavage fluid collection and analysis

The mice were euthanized by CO<sub>2</sub> inhalation. Immediately thereafter, a midline neck incision was made and the trachea was cannulated. The lungs were lavaged 3 times with 1.0 mL PBS containing 1% FCS and 0.5 mmol/L EDTA. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 400g for 5 min-

utes at 4°C and resuspended in 200  $\mu$ L PBS containing 1% FCS and 0.5 mol/L EDTA. Lysis of red blood cells was carried out through use of RBC lysis buffer (Sigma, St Louis, Mo), according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of  $5 \times 10^4$  cells were stained with Giemsa-Diff-Quick (Dade Diagnostics of PR, Inc, Aguada, Puerto Rico), and differential cell counts were determined.

### Peripheral blood collection and analysis

Peripheral blood samples were collected in heparinized tubes (Becton Dickinson, Franklin Lakes, NJ) by collecting pooled blood from the inguinal artery of anesthetized IL-13-treated or saline solution-treated mice. Blood smears were stained with Giemsa-Diff-Quick, and differential cell counts were performed. In addition, eosinophil levels were directly determined with Dicombe's stain.<sup>31</sup>

### Bone marrow collection and analysis

The bone marrow from 1 femur was isolated by flushing 5.0 mL PBS, 1% FCS, and 0.5 mmol/L EDTA with a 23-gauge needle. The recovered bone marrow was centrifuged at 400g for 5 minutes at 4°C and resuspended in 1.0 mL PBS containing 1% FCS and 0.5 mmol/L EDTA. Lysis of red blood cells was carried out through use of RBC lysis buffer according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of  $5 \times 10^4$  cells were stained with Giemsa-Diff-Quick combination, and differential cell counts were performed.

### Mucus production analysis

Lung tissue samples were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), embedded in paraffin, cut into 5- $\mu$ m sections, and fixed to positively charged slides. Periodic-acid Schiff reaction staining (Poly Scientific R&D Corp, Bay Shore, NY) was then performed on the tissue sections, according to the manufacturer's recommendations. A 1.0  $\times$  1.0-cm ocular grid was used to quantify the percentage of pulmonary epithelium that was producing mucus. Specifically, 300 to 500 linear gradations (representing 5 mm of epithelium) were counted from a random selection of medium-sized airways. Each result is expressed as the percentage of epithelium positive for the mucus stain.

### Eosinophil staining in tissue

Lung tissue samples were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), embedded in paraffin, cut into 5- $\mu$ m sections, and fixed to positively charged slides. Eosinophils in the tissue were differentially stained through use of an antiserum against murine major basic protein (MBP), as described previously.<sup>32</sup> In brief, tissue endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol; this was followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rabbit antimurine MBP antibody (1:16,000; a kind gift of J. Lee, Mayo Clinic, Scottsdale, Ariz) overnight at 4°C; this was followed by 1:200 dilution of biotinylated goat antirabbit IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories, Burlingame, Calif) for 30 minutes each. The slides were further treated with nickel diaminobenzidine/cobalt chloride solution (to form a black precipitate) and counterstained with nuclear fast red. Quantification of immunoreactive cells was performed by morphometric analysis through use of the Metamorph Imaging System (Universal Imaging Corporation, West Chester, Pa), as described previously.<sup>33</sup> The lung sections were taken from the same lobe in each mouse, and at least 3 to 4 random sections per mouse were analyzed. Through the use of digital image capture, tissue regions associated with medium-sized bronchioles were quantified for the total MBP-positive cell numbers relative to the total tissue area. Calculated eosinophil levels are expressed in terms of cells per square millimeter.

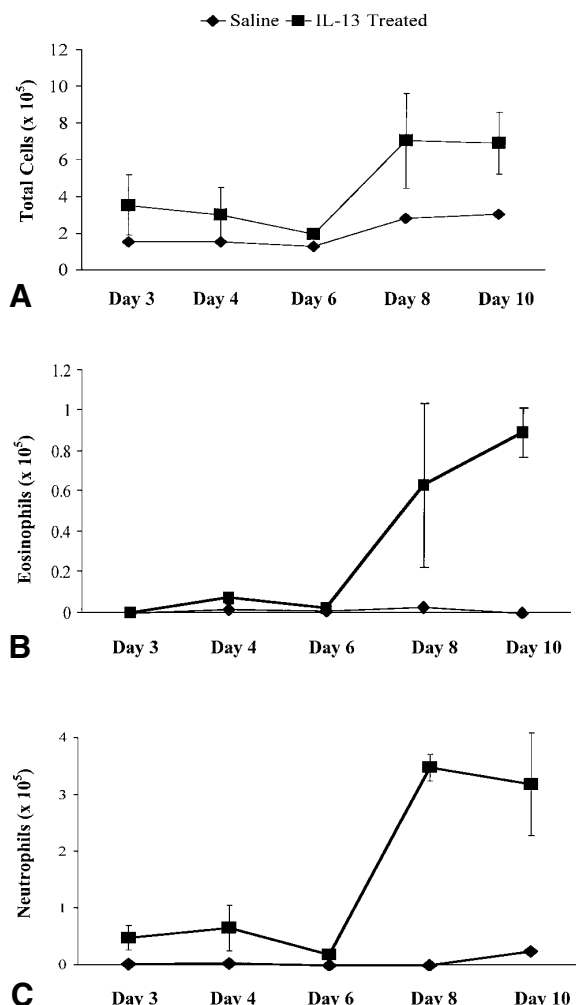


**FIG 1.** Kinetic analysis of IL-13-induced leukocytes in BALF. Mice received intranasal treatment with 10  $\mu$ g/day of either IL-13 or control saline solution. The total leukocyte (A), eosinophil (B), and neutrophil (C) levels in BALF 18 hours after the day of cytokine administration are shown. Results are expressed as means  $\pm$  SDs. Because of the small number of mice examined at each time point, statistical analysis was not performed for this initial kinetic analysis.

## Statistical analysis

Data are expressed as means  $\pm$  SDs. The statistical significance when sets of mice were compared was determined through use of the Student *t* test.

## RESULTS

### Development of IL-13-induced pulmonary inflammation

To examine the mechanism of IL-13-induced airway eosinophilia, it was necessary first to establish the conditions for IL-13 induction of airway inflammation. Initially, eosinophil infiltration into BALF was measured as an indicator for the induction of airway inflammation. Ten micrograms of IL-13 or saline solution was intranasally

administered daily to lightly anesthetized wild-type Balb/C mice for 3, 4, 6, 8, and 10 days. BALF, bone marrow, and peripheral blood cell counts were analyzed 16 to 20 hours after each dose. Although the total number of cells in BALF also increased (Fig 1, A), the composition of the cell population showed a preferential recruitment of eosinophils into BALF. For example, eosinophils increased from less than 1% in control mice to 13% in mice treated with IL-13 for 10 consecutive days ( $P = .04$ ). From a baseline level of approximately  $10^3$ , the number of eosinophils in BALF increased to  $9.0 \times 10^4$  (Fig 1, B). Of note, after 8 doses, an infiltration of neutrophils in BALF was seen; this represented 53% of the total cells in BALF (Fig 1, C). No significant variation in number or type of cells in the bone marrow or peripheral blood was observed during the kinetic study. For example, eosinophil differentials in the bone marrow were  $4.4\% \pm 1.3\%$  and  $4.8\% \pm 2.8\%$  (mean  $\pm$  SD;  $n = 5$ ) in mice treated with 10 doses of saline solution and mice treated with 10 doses of IL-13, respectively. In addition, peripheral blood eosinophil differentials were  $0.7\% \pm 0.8\%$  and  $1.4\% \pm 2\%$  (mean  $\pm$  SD;  $n = 5$ ) after treatment with saline solution and IL-13, respectively. Consistent with this, absolute peripheral blood eosinophil counts were  $3.2 \times 10^4$  and  $3.6 \times 10^4$  cells/mL in saline solution- and IL-13-treated mice, respectively. Treatment of mice with 10  $\mu$ g of IL-13 for 10 consecutive days was therefore used for subsequent analysis.

### IL-5 is required for IL-13-induced eosinophilia

To examine the effect of IL-5 on IL-13-induced recruitment of eosinophils into the lung, IL-13 was next administered to IL-5 gene-targeted mice. The leukocyte differential counts in the bone marrow and peripheral blood were determined. The peripheral blood cell differentials and the bone marrow differentials showed no discernable differences between saline solution- and IL-13-treated mice (data not shown). IL-5 gene-targeted mice treated with saline solution had BALF eosinophil levels below the detection limit; their IL-13-treated counterparts had  $3.8 \pm 6.6 \times 10^3$  eosinophils in BALF (Fig 2, A). In contrast, wild-type mice had  $2.05 \pm 2.49 \times 10^3$  and  $7.95 \pm 5.46 \times 10^4$  eosinophils in BALF in response to saline solution and IL-13, respectively. It is interesting to note that the percent of eosinophils in BALF in response to IL-13 was  $0.92\% \pm 1.25\%$  in IL-5 gene-targeted mice; this compared with  $8.49\% \pm 3.16\%$  in wild-type mice (Fig 2, B).

We next examined eosinophil levels in the peribronchial region of the lung tissue after IL-13 treatment of IL-5 gene-targeted mice (Fig 3). Eosinophils were identified by immunostaining with anti-MBP (Fig 4, A-D). Consistent with the impaired eosinophil recruitment into BALF, IL-13-induced lung eosinophilia was markedly attenuated in the absence of IL-5 ( $23 \pm 26$  vs  $108 \pm 39$  eosinophils/mm<sup>2</sup> in IL-13-treated IL-5-deficient and wild-type mice, respectively). Thus IL-13-induced eosinophil recruitment into the lung is largely dependent on IL-5.

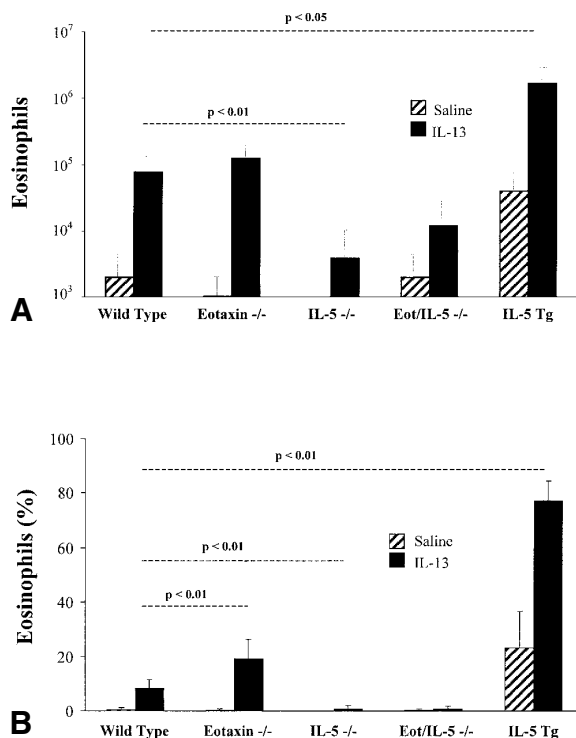


**FIG 2.** IL-13-induced eosinophil counts in BALF. Mice were treated for 10 days with IL-13 (solid) or saline solution (hatched). The total eosinophil levels (A) and eosinophil percentages (B) in BALF of wild-type (n = 10), eotaxin -/- (n = 14), IL-5 -/- (n = 9), eotaxin/IL-5-double-deficient (*Eot/IL-5* -/-; n = 10), and IL-5 transgenic (*Tg*; n = 6) mice were determined. The results are data pooled from 3 experiments and are expressed as means  $\pm$  SDs.

### IL-5 enhances IL-13 induction of eosinophilia

We were next interested in determining whether overproduction of IL-5 by T cells, a process that occurs in patients with asthma,<sup>3</sup> influences IL-13-induced airway eosinophilia. We therefore subjected mice carrying the IL-5 transgene (under the control of the T cell-specific CD2 promoter) to IL-13 treatment. Transgenic mice receiving IL-13 were found to develop a 2-fold increase in total cells in BALF in comparison with wild-type mice (data not shown). Interestingly, IL-13-treated IL-5 transgenic mice developed a 20-fold increase in BALF eosinophils in comparison with wild-type mice ( $1.71 \pm 1.18 \times 10^6$  and  $7.95 \pm 5.46 \times 10^4$ , respectively; Fig 2, A). Furthermore, IL-5 transgenic mice had a remarkable level of airway eosinophilia, with  $77.5\% \pm 7.1\%$  eosinophils in BALF (Fig 2, B and Fig 4, E and F). It is worth noting that IL-5 transgenic mice treated with saline solution had levels of eosinophils that were higher than saline solution- or IL-13-treated wild-type mice (Figs 2 and 3).

### Eotaxin regulates IL-13-induced eosinophil distribution in the lung

We were next interested in examining the role of eotaxin in the recruitment of eosinophils into the lung. IL-13 was therefore administered to eotaxin gene-targeted

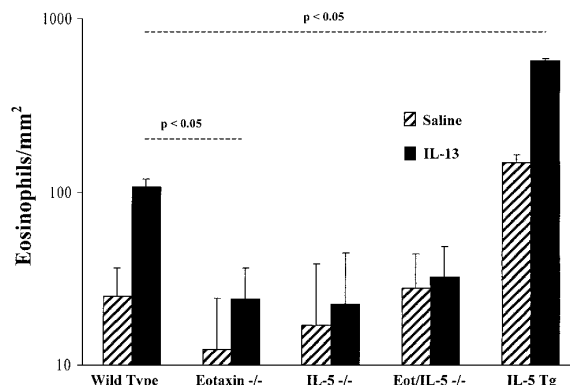


**FIG 3.** IL-13-induced lung tissue eosinophilia. Mice were treated for 10 consecutive days with saline solution (hatched) or IL-13 (solid). The level of eosinophils in the lung tissue was determined by morphometric analysis from wild-type (n = 5), eotaxin -/- (n = 8), IL-5 -/- (n = 8), eotaxin/IL-5-double-deficient (*Eot/IL-5* -/-; n = 10), and IL-5 transgenic (*Tg*; n = 4) mice. The results are data pooled from 2 representative experiments and are expressed as means  $\pm$  SDs.

and wild-type mice. As in the treatment of wild-type and IL-5 gene-targeted mice, the leukocyte differential counts in the bone marrow and peripheral blood showed no IL-13-induced differences (data not shown). Surprisingly, however, IL-13 treatment induced a percentage of BALF eosinophils that was 2-fold higher ( $P < .01$ ) in eotaxin gene-targeted mice than in wild-type mice (Fig 2, B). In wild-type mice, the percentage of eosinophils was  $0.77\% \pm 0.82\%$  for saline solution-treated mice and  $8.49\% \pm 3.17\%$  after IL-13 treatment. In eotaxin gene-targeted mice, the percentage of eosinophils was  $0.60\% \pm 0.47\%$  and  $19.35\% \pm 7.17\%$  after treatment with saline solution and IL-13, respectively. Analysis of total eosinophil levels in BALF in response to IL-13 revealed that eosinophil recruitment into BALF was independent of eotaxin ( $7.95 \pm 5.46 \times 10^4$  and  $1.26 \pm 0.74 \times 10^5$  eosinophils for IL-13-treated wild-type and eotaxin-deficient mice, respectively; Fig 2, A).

We next examined the tissue level of eosinophils in the lungs after IL-13 treatment. Even though BALF eosinophilia was not reduced in eotaxin-deficient mice, the same mice had a marked deficiency of airway tissue eosinophilia in comparison with wild-type mice ( $24.41 \pm 24.86$  vs  $108 \pm 39.0$  eosinophils/mm<sup>2</sup>; Fig 3 and Fig 4, H and B). In fact, the level of airway eosinophils was similar to that seen in IL-5-deficient mice. Finally, mice deficient in both IL-5 and eotaxin were examined. These double-deficient mice were found to have impaired eosinophil recruitment into both BALF and tissue (Figs 2 and 3), which is consistent with results in IL-5 gene-deficient mice.

### Mucus production is not dependent on IL-5 or eotaxin

We were next interested in determining whether IL-13-induced mucus production was influenced by the level of eosinophils in the lung (Figs 5 and 6). Mucus

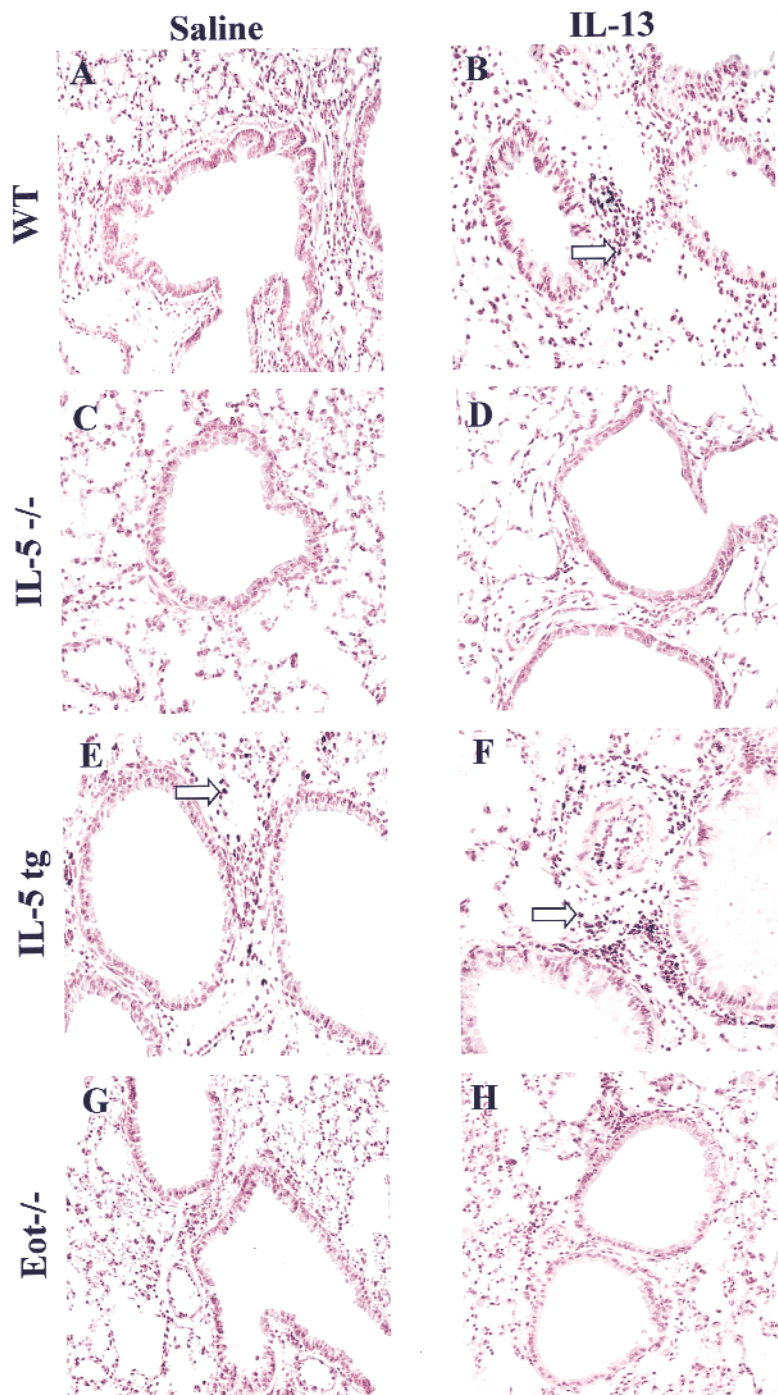


**FIG 4.** Photomicrographs of saline solution- and IL-13-treated lung sections stained with anti-MBP. Mice were treated for 10 consecutive days with saline solution or IL-13, and representative sections from wild-type (**A, B**), IL-5  $-/-$  (**C, D**), IL-5 transgenic (*tg*; **E, F**), and eotaxin  $-/-$  (**G, H**) mice are shown. In panels **A** through **H**, sections are stained with anti-MBP and eosinophils are identified as black-stained cells. The arrows point to representative MBP-positive eosinophils. Eosinophils are observable only in saline solution-treated IL-5 transgenic (**E**), IL-13 treated wild-type (**B**), and IL-5 transgenic (**F**) mice.

production was detected by staining with the periodic-acid Schiff reaction, as shown in Fig 6. In wild-type mice, IL-13 induced an increase in mucus production >25-fold in comparison with saline solution ( $26\% \pm 17\%$  and  $0.92\% \pm 0.67\%$ , respectively). In eotaxin gene-targeted

mice treated with IL-13, there was an increase in mucus production from  $0.41\% \pm 0.45\%$  to  $46\% \pm 19\%$ . Likewise, IL-13 delivery to IL-5 transgenic mice increased mucus production from  $0.30\% \pm 0.41\%$  to  $37\% \pm 16\%$ . Finally, IL-13 treatment of IL-5 gene-targeted

mice resulted in mucus production in  $38\% \pm 11\%$  of the airway epithelium. Induction of IL-13-induced airway mucus production was thus not influenced by either IL-5 or eotaxin, which suggests no association between mucus production and airway eosinophilia.

## DISCUSSION

The potent inflammatory actions of IL-13 have identified this molecule as a potential regulator of multiple aspects of asthma, including airway hyperresponsiveness, eosinophilia, and mucus overproduction.<sup>4</sup> Although IL-13 has been shown to be a powerful inducer of chemokines,<sup>12,13,34</sup> the mechanism of IL-13-induced lung eosinophilia has not been established.

In the present study, we used a mouse model system to delineate the mechanism by which IL-13 induces eosinophilia. First, our results establish that IL-13 induces airway eosinophilia independently of effects on eosinophilopoiesis or circulating levels of eosinophils; this indicates that IL-13 (in this model system) is primarily mediating a local effect in the lungs. Consistent with this finding, IL-13 has been demonstrated to be a potent inducer of eosinophil adhesion molecules, such as vascular cell adhesion molecule 1, and eosinophil-active chemokines.<sup>35</sup> Second, we demonstrate that IL-13-induced eosinophilia in the lung and BALF is largely dependent on IL-5. Interestingly, IL-5 and IL-13 are often coproduced by  $T_H2$  cells, which provides a scenario for the cooperation of these 2 cytokines.<sup>4</sup> Third, we establish that IL-13-induced airway eosinophilia is influenced by eotaxin; however, unlike IL-5, eotaxin is not required for airway eosinophilia, inasmuch as eosinophils accumulate in the airway lumen in eotaxin-deficient mice. Rather, eotaxin appears to play a role in regulating the distribution of eosinophils within the lungs, because the tissue level of eosinophils is attenuated in the absence of eotaxin. This finding suggests that eotaxin plays a role in retaining recruited eosinophils in the lungs. These last 2 findings, taken together, indicate that the effect of IL-13 on inducing airway eosinophilia is not a direct one; rather, it involves both eotaxin and IL-5. Finally, we establish that IL-13-induced mucus secretion occurs independently of eotaxin and IL-5, which provides strong evidence that mucus secretion is dissociated from eosinophilia. This is consistent with a previous report that  $T_H2$ -induced airway mucus production is dependent on IL-4R $\alpha$  but not on eosinophils.<sup>36</sup> The mucus production is likely to be explained by direct signaling of IL-13 in respiratory epithelial cells via the IL-4R $\alpha$ /IL-13R $\alpha_1$  molecules known to be expressed by these cells.<sup>35,37</sup> In addition, IL-13 has been shown to stimulate mucus secretion by activating neutrophils, at least in part.<sup>38</sup> Consistent with this, we have found that IL-13 administration induces substantial airway neutrophilia (Fig 1, C). Although this was not the focus of the current study, IL-13 also activates neutrophils.<sup>39</sup>

IL-5 and eotaxin have previously been shown to be critical regulators of eosinophil trafficking and accumulation

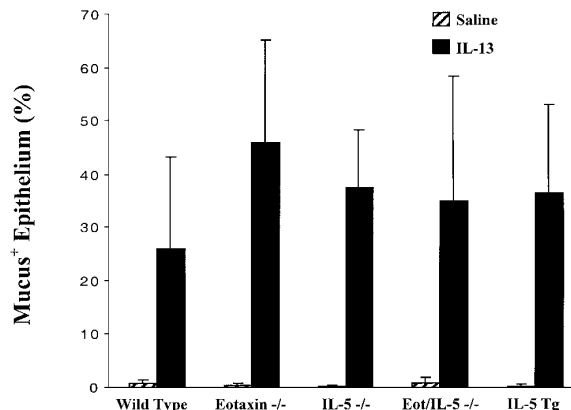


**FIG 5.** IL-13-induced mucus production. Mice were treated for 10 consecutive days with saline solution (hatched) or IL-13 (solid), and the percentage of epithelium positive for mucus (after periodic-acid Schiff staining) is shown for wild-type ( $n = 5$ ), eotaxin  $-/-$  ( $n = 6$ ), IL-5  $-/-$  ( $n = 8$ ), eotaxin/IL-5-double-deficient (*Eot/IL-5*  $-/-$ ;  $n = 6$ ), and IL-5 transgenic (*Tg*;  $n = 5$ ) mice. The results are data pooled from 2 representative experiments and are expressed as means  $\pm$  SDs.

into tissue at baseline and during antigen-driven inflammatory responses.<sup>18,23,29</sup> IL-13 is thought to collaborate with this pathway by regulating eotaxin expression.<sup>13</sup> IL-4 and IL-13 share the ability to induce eotaxin *in vivo*<sup>12,13,40</sup> in a variety of cells, including respiratory epithelium<sup>41,42</sup> and nasal and dermal fibroblasts,<sup>34,43</sup> by an apparently STAT-6-dependent pathway.<sup>44-46</sup> IL-13 appears more potent than IL-4, inasmuch as inhibition of IL-13 with a neutralizing antibody markedly attenuates the eotaxin-inducing activity of  $T_H2$  cell supernatants.<sup>13</sup> In our study, the level of IL-13-mediated eosinophil accumulation was low in comparison with antigen-driven allergic inflammatory responses (experimental asthma models).<sup>20,27</sup> This might reflect the requirement for the upregulation of both eotaxin and IL-5 and their synergistic interactions for maximal eosinophilic responses. In particular, IL-5 promotes an expansion of circulating eosinophils in response to antigen provocation, whereas eotaxin subsequently sequesters eosinophils into tissues.<sup>3,47</sup>

An unexpected result in this study was the finding that eotaxin-deficient mice had a major impairment in IL-13-induced eosinophilia in the lung tissue but not in BALF. It is interesting to speculate that this reflects the ability of eotaxin to retain recruited eosinophils in the lung tissue. Eosinophils might be initially recruited into the lung in response to local overproduction of IL-5 and other eosinophil-active chemokines (eg, RANTES). Chemokines, which have long been known to be potent leukocyte chemoattractants, have been recently recognized to regulate the spatial distribution of leukocytes in secondary lymphoid compartments and inflammatory sites.<sup>48-50</sup> For example, eotaxin induces both firm adhesion and detachment of eosinophils to membrane integrins and extracellular matrix components,<sup>51-53</sup> thus providing a scenario for eotaxin-dependent tissue retention. Such changes might be mediated by eotaxin-induced

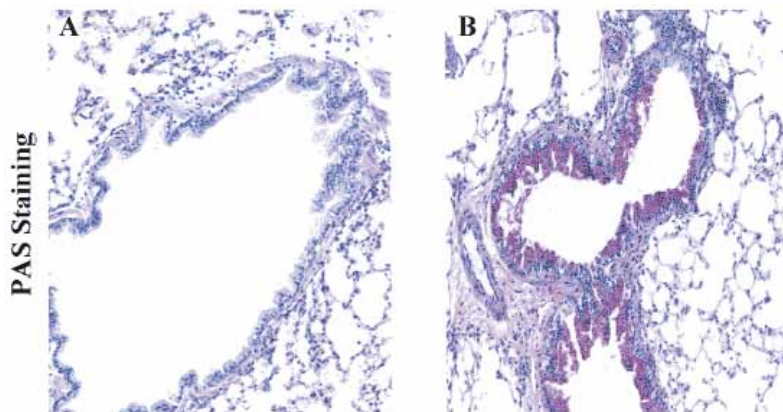


**FIG 6.** Photomicrographs of saline solution- and IL-13-treated lung sections stained for mucus. Mice were treated for 10 consecutive days with saline solution (**A**) or IL-13 (**B**); representative lung sections from wild-type mice are shown. Sections were stained with periodic-acid Schiff stain, and mucus-expressing cells are identified as purple-staining cells, seen primarily in the IL-13 treated mice.

changes in the expression and activation of adhesion molecules on the cell surface of eosinophils.<sup>2,54</sup> The recently recognized ability of eotaxin to rapidly induce downregulation of its own receptor (CCR3)<sup>55</sup> and a subsequent inhibitory chemotactic response (M. E. Rothenberg and N. Zimmermann, unpublished data) provides an additional mechanism for chemokine-mediated tissue retention.

Taken together, these results indicate that IL-13-induced eosinophilia in the lung is differentially regulated by both IL-5 and eotaxin. Integrated signals involving T<sub>H</sub>2 cells and their cytokines (IL-5 and IL-13), as well as chemokines, are likely to be involved in the immunopathogenesis of the airway inflammation seen in asthma. Currently, therapeutic intervention strategies in patients with asthma are being designed to neutralize single cytokines (eg, anti-IL5); the results of the current study suggest that strategies that coinhibit multiple cytokines might provide additional benefit.

We are grateful to Dr Debra D. Donaldson and the Genetics Institute (Cambridge, Mass) for a generous supply of murine IL-13 and to Drs Jamie and Nancy Lee (Mayo Clinic, Scottsdale, Ariz) for a generous supply of anti-MBP.

## REFERENCES

1. Rothenberg ME. Eosinophilia. *N Engl J Med* 1998;338:1592-600.
2. Bochner BS, Schleimer RP. Mast cells, basophils, and eosinophils: distinct but overlapping pathways for recruitment. *Immunol Rev* 2001;179:5-15.
3. Hamelmann E, Gelfand EW. IL-5-induced airway eosinophilia—the key to asthma? *Immunol Rev* 2001;179:182-91.
4. Wills-Karp M. IL-12/IL-13 axis in allergic asthma. *J Allergy Clin Immunol* 2001;107:9-18.
5. Humbert M, Durham SR, Kimmitt P, Powell N, Assoufi B, Pfister R, et al. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin Immunol* 1997;99:657-65.
6. Akdis M, Akdis CA, Weigl L, Disch R, Blaser K. Skin-homing, CLA+ memory T cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated cytokine pattern: IgG4 counter-regulation by CLA+ memory T cells. *J Immunol* 1997;159:4611-9.
7. Katagiri K, Itami S, Hatano Y, Takayasu S. Increased levels of IL-13 mRNA, but not IL-4 mRNA, are found in vivo in peripheral blood mononuclear cells (PBMC) of patients with atopic dermatitis (AD). *Clin Exp Immunol* 1997;108:289-94.
8. Ying S, Meng Q, Barata LT, Robinson DS, Durham SR, Kay AB. Associations between IL-13 and IL-4 (mRNA and protein), vascular cell adhesion molecule-1 expression, and the infiltration of eosinophils, macrophages, and T cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Immunol* 1997;158:5050-7.
9. Pawankar RU, Okuda M, Hasegawa S, Suzuki K, Yssel H, Okubo K, et al. Interleukin-13 expression in the nasal mucosa of perennial allergic rhinitis. *Am J Respir Crit Care Med* 1995;152:2059-67.
10. Pawankar R, Okuda M, Yssel H, Okumura K, Ra C. Nasal mast cells in perennial allergic rhinitis exhibit increased expression of the FcεRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J Clin Invest* 1997;99:1492-9.
11. al Ghamdi K, Ghaffar O, Small P, Frenkel S, Hamid Q. IL-4 and IL-13 expression in chronic sinusitis: relationship with cellular infiltrate and effect of topical corticosteroid treatment. *J Otolaryngol* 1997;26:160-6.
12. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;103:779-88.
13. Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, et al. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* 1999;162:2477-87.
14. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258-61.
15. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261-3.
16. Rankin SM, Conroy DM, Williams TJ. Eotaxin and eosinophil recruitment: implications for human disease. *Mol Med Today* 2000;6:20-7.
17. Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 1995;182:1169-74.
18. Mould AW, Ramsay AJ, Matthaai KI, Young IG, Rothenberg ME, Foster PS. The effect of IL-5 and eotaxin expression in the lung on eosinophil trafficking and degranulation and the induction of bronchial hyperactivity. *J Immunol* 2000;164:2142-50.
19. Rothenberg ME. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *Am J Respir Cell Mol Biol* 1999;21:291-5.
20. Rothenberg ME, MacLean JA, Pearlman E, Luster AD, Leder P. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp Med* 1997;185:785-90.
21. Campbell EM, Kunkel SL, Strieter RM, Lukacs NW. Temporal role of

- chemokines in a murine model of cockroach allergen-induced airway hyperactivity and eosinophilia. *J Immunol* 1998;161:7047-53.
22. Rothenberg ME, Ownbey R, Mehlhop PD, Loissele PM, Van de Rijn M, Bonventre JV, et al. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice. *Mol Med* 1996;2:334-48.
23. Mould AW, Matthaei KI, Young IG, Foster PS. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J Clin Invest* 1997;99:1064-71.
24. Luttmann W, Knoechel B, Foerster M, Matthys H, Virchow JC, Kroegel C. Activation of human eosinophils by IL-13. Induction of CD69 surface antigen, its relationship to messenger RNA expression, and promotion of cellular viability. *J Immunol* 1996;157:1678-83.
25. Luttmann W, Matthies T, Matthys H, Virchow JC. Synergistic effects of interleukin-4 or interleukin-13 and tumor necrosis factor- $\alpha$  on eosinophil activation in vitro. *Am J Respir Cell Mol Biol* 1999;20:474-80.
26. Horie S, Okubo Y, Hossain M, Sato E, Nomura H, Koyama S, et al. Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. *Intern Med* 1997;36:179-85.
27. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195-201.
28. Huang WW, Garcia-Zepeda EA, Sauty A, Oettgen HC, Rothenberg ME, Luster AD. Molecular and biological characterization of the murine leukotriene B<sub>4</sub> receptor expressed on eosinophils. *J Exp Med* 1998;188:1063-74.
29. Mishra A, Hogan SP, Lee JJ, Foster PS, Rothenberg ME. Fundamental signals regulate eosinophil homing to the gastrointestinal tract. *J Clin Invest* 1999;103:1719-27.
30. Mishra A, Hogan SP, Brandt EB, Rothenberg ME. An etiological role for aeroallergens and eosinophils in experimental esophagitis. *J Clin Invest* 2001;107:83-90.
31. Discombe G. Criteria of eosinophilia. *Lancet* 1946;1:195.
32. Matthews AN, Friend DS, Zimmermann N, Sarafi MN, Luster AD, Pearlman E, et al. Eotaxin is required for the baseline level of tissue eosinophils. *Proc Natl Acad Sci U S A* 1998;95:6273-8.
33. Hogan SP, Mishra A, Brandt EB, Royalty MP, Pope SM, Zimmermann N, et al. A pathological function for eotaxin and eosinophils in eosinophilic gastrointestinal inflammation. *Nat Immunol* 2001;2:353-60.
34. Terada N, Hamano N, Nomura T, Numata T, Hirai K, Nakajima T, et al. Interleukin-13 and tumour necrosis factor- $\alpha$  synergistically induce eotaxin production in human nasal fibroblasts. *Clin Exp Allergy* 2000;30:348-55.
35. Bochner BS, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol* 1995;154:799-803.
36. Cohn L, Homer RJ, MacLeod H, Mohrs M, Brombacher F, Bottomly K. Th2-induced airway mucus production is dependent on IL-4R $\alpha$ , but not on eosinophils. *J Immunol* 1999;162:6178-83.
37. Ceponis PJ, Botelho F, Richards CD, McKay DM. Interleukins 4 and 13 increase intestinal epithelial permeability by a phosphatidylinositol 3-kinase pathway. Lack of evidence for STAT 6 involvement. *J Biol Chem* 2000;275:29132-7.
38. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, et al. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L134-L140.
39. Zaitu M, Hamasaki Y, Matsuo M, Kukita A, Tsuji K, Miyazaki M, et al. New induction of leukotriene A(4) hydrolase by interleukin-4 and interleukin-13 in human polymorphonuclear leukocytes. *Blood* 2000;96:601-9.
40. Rothenberg ME, Luster AD, Leder P. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc Natl Acad Sci U S A* 1995;92:8960-4.
41. Garcia-Zepeda EA, Rothenberg ME, Ownbey RT, Celestin J, Leder P, Luster AD. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat Med* 1996;2:449-56.
42. Stellato C, Matsukura S, Fal A, White J, Beck LA, Proud D, et al. Differential regulation of epithelial-derived C-C chemokine expression by IL-4 and the glucocorticoid budesonide. *J Immunol* 1999;163:5624-32.
43. Mochizuki M, Schroder J, Christophers E, Yamamoto S. IL-4 induces eotaxin in human dermal fibroblasts. *Int Arch Allergy Immunol* 1999;120 Suppl 1:19-23.
44. Matsukura S, Stellato C, Plitt JR, Bickel C, Miura K, Georas SN, et al. Activation of eotaxin gene transcription by NF- $\kappa$ B and STAT6 in human airway epithelial cells. *J Immunol* 1999;163:6876-83.
45. Zimmermann N, Hogan SP, Mishra A, Brandt EB, Bodette TR, Pope SM, et al. Murine eotaxin-2: a constitutive eosinophil chemokine induced by allergen challenge and IL-4 overexpression. *J Immunol* 2000;165:5839-46.
46. Hoeck J, Woisetschlager M. STAT6 mediates eotaxin-1 expression in IL-4 or TNF- $\alpha$ -induced fibroblasts. *J Immunol* 2001;166:4507-15.
47. Foster PS, Mould AW, Yang M, Mackenzie J, Mattes J, Hogan SP, et al. Elemental signals regulating eosinophil accumulation in the lung. *Immunol Rev* 2001;179:173-81.
48. Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH, et al. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 1996;98:2332-45.
49. Gonzalo JA, Lloyd CM, Wen D, Albar JP, Wells TN, Proudfoot A, et al. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* 1998;188:157-67.
50. Lukacs NW, Oliveira SH, Hogaboam CM. Chemokines and asthma: redundancy of function or a coordinated effort? *J Clin Invest* 1999;104:995-9.
51. Weber C, Kitayama J, Springer TA. Differential regulation of B1 and B2 integrin avidity by chemoattractants in eosinophils. *Proc Natl Acad Sci U S A* 1996;93:10939-44.
52. Yuan Q, Austen KF, Friend DS, Heidtman M, Boyce JA. Human peripheral blood eosinophils express a functional c-kit receptor for stem cell factor that stimulates very late antigen 4 (VLA-4)-mediated cell adhesion to fibronectin and vascular cell adhesion molecule 1 (VCAM-1). *J Exp Med* 1997;186:313-23.
53. Tachimoto H, Burdick MM, Hudson SA, Kikuchi M, Konstantopoulos K, Bochner BS. CCR3-active chemokines promote rapid detachment of eosinophils from VCAM-1 in vitro. *J Immunol* 2000;165:2748-54.
54. Hohki G, Terada N, Hamano N, Kitaura N, Nakajima T, Yoshie O, et al. The effects of eotaxin on the surface adhesion molecules of endothelial cells and on eosinophil adhesion to microvascular endothelial cells. *Biochem Biophys Res Commun* 1997;241:136-41.
55. Zimmermann N, Konkright JJ, Rothenberg ME. CC chemokine receptor-3 undergoes prolonged ligand-induced internalization. *J Biol Chem* 1999;274:12611-8.