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IL-5 Promotes Eosinophil Trafficking to the Esophagus¹

Anil Mishra,* Simon P. Hogan,[†] Eric B. Brandt,* and Marc E. Rothenberg^{2*}

Eosinophil infiltration into the esophagus occurs in a wide range of diseases; however, the underlying pathophysiological mechanisms involved are largely unknown. We now report that the Th2 cytokine, IL-5, is necessary and sufficient for the induction of eosinophil trafficking to the esophagus. We show that transgenic mice overexpressing IL-5 under the control of a T cell (CD2) or a small intestinal enterocyte (fatty acid-binding protein) promoter have markedly increased eosinophil numbers in the esophagus. For example, esophageal eosinophil levels are 1.9 ± 0.9 and 121 ± 14 eosinophils/mm² in wild-type and CD2-IL-5-transgenic mice, respectively. Consistent with this effect being mediated by a systemic mechanism, pharmacological administration of IL-5 via a miniosmotic pump in the peritoneal cavity resulted in blood and esophageal eosinophilia. To examine the role of IL-5 in oral Ag-induced esophageal eosinophilia, eosinophilic esophagitis was induced by allergen exposure in IL-5-deficient and wild-type mice. Importantly, IL-5-deficient mice were resistant to eosinophilic esophagitis. Finally, we examined the role of eotaxin when IL-5 was overproduced in vivo. Esophageal eosinophil levels in CD2-IL-5-transgenic mice were found to decrease 15-fold in the absence of the eotaxin gene; however, esophageal eosinophil numbers in eotaxin-deficient IL-5-transgenic mice still remained higher than wild-type mice. In conclusion, these studies demonstrate a central role for IL-5 in inducing eosinophil trafficking to the esophagus. *The Journal of Immunology*, 2002, 168: 2464–2469.

One of the unique properties of the esophagus compared with other gastrointestinal segments is that it normally does not contain resident eosinophils (1). Despite this, the accumulation of eosinophils in the esophagus is a commonly observed medical problem in patients with diverse diseases, including gastroesophageal reflux, eosinophilic esophagitis, eosinophilic gastroenteritis, and parasitic infections (2–6). The accumulation of eosinophils in the esophagus has been associated with allergic responses; for example, patients with eosinophilic esophagitis have a high rate of atopy, and their clinical symptoms and eosinophilic infiltrations are ameliorated by an elemental diet or antiinflammatory therapy (cromoglycate or glucocorticoids) (7, 8). Although the role of allergens in the induction of eosinophilia in the esophagus has been debated (9), recent experimental studies have established a linkage. Exposure of anesthetized mice to repeated challenges of aeroallergen (e.g., extracts of *Aspergillus fumigatus*) using a protocol to induce allergic airway inflammation promotes marked eosinophilic esophagitis (10). The allergen-induced esophageal eosinophilia is accompanied by intraepithelial eosinophils, extracellular granule deposition, and epithelial cell hyperplasia, features that mimic the pathophysiological changes observed in individuals with various forms of eosinophilic esoph-

agitis (9, 10). Importantly, the eosinophilic inflammation occurs in the lungs and esophagus, but not the stomach or intestine, demonstrating an intimate immunological connection between Th2-associated allergic responses in the lung and esophagus. Of the cytokines produced by Th2 cells, IL-5 is the most specific for eosinophils. IL-5 induces eosinophil growth, differentiation, activation, and survival of eosinophils and primes eosinophils to respond to chemoattractants such as eotaxin, an eosinophil-selective CC chemokine (11–13). Additionally, IL-5 has been shown to be necessary for pulmonary and esophageal eosinophilia in response to respiratory allergen challenge (10). We now further examine the mechanism of eosinophilic esophagitis by examining the consequences of overexpressing IL-5 by transgenic or pharmacological administration. In addition, we examine the requirement of IL-5 for the induction of eosinophilic esophagitis associated with oral Ag-induced eosinophilic gastroenteritis. Finally, we test the role of eotaxin in regulating eosinophilic esophagitis when IL-5 is overproduced.

Materials and Methods

Mice

Specific pathogen-free 129SvEv and BALB/c mice (8–10 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Eotaxin-deficient inbred mice of the BALB/c background were maintained with age- and sex-matched controls from Taconic Farms (Germantown, PA), as described (14). Mice transgenic for IL-5 (on a CBA background) were originally obtained from C. Sanderson (Institute for Child Health Research, Perth, Australia) and were backcrossed into the BALB/c background and analyzed after 10 backcrosses (15, 16).

Generation of eotaxin-deficient IL-5-transgenic mice

IL-5-transgenic mice (BALB/c) carrying the eotaxin wild-type or gene-targeted allele were generated by breeding F₁ littermates (from eotaxin gene-targeted mice crossed with IL-5-transgenic mice) with eotaxin gene-targeted mice and screening tail DNA by Southern analysis for the eotaxin gene-targeted allele and the IL-5 transgene (16).

IL-5 delivery

Mice were anesthetized by inhalation of isoflurane, and their ventral skin was shaved and washed with hibitane. Miniosmotic pumps (ALZA Pharmaceuticals, Palo Alto, CA) containing 500 and 1000 pmol/kg body weight of human IL-5 (a kind gift of R. Egen, Schering Plough, Kenilworth, NJ)

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or control vehicle (10 mM PBS/0.1% BSA, pH 7.4) were i.p. implanted surgically under sterile conditions, and the wound was sealed as described (17, 18). The miniosmotic pump delivers IL-5 or control vehicle in the mice peritoneum at the rate of 1 μ l/h (i.e., \sim 2 or 4 pmol IL-5/kg body weight/h) for 8 days. After 8 days, all mice were sacrificed, and their blood and esophageal tissue were analyzed for eosinophil levels.

Generation of intestine eotaxin and IL-5-transgenic mice

Oligonucleotides containing *Bam*HI sites were ligated to both ends of a 330-bp fragment containing the coding region of the murine eotaxin cDNA. A 415-bp fragment of the entire coding region of murine IL-5 cDNA was amplified by PCR incorporating an improved Kozak consensus sequence and *Bam*HI restriction sites on both ends. Both cDNAs were ligated into the *Bam*HI sites of the pBSIF1178-hGHpgkNeo plasmid (19, 20), which contained the rat fatty acid-binding protein (fabpi)³ promoter and the human growth hormone gene. The details of these mice are described in another reference (21).

OVA treatment of mice

A mouse model of allergic gastrointestinal disease was established using methods previously described (22). In brief, mice were sensitized by i.p. injection with OVA (50 mg) and alum (1 mg) in 0.9% sterile saline on day 0. On days 12 and 15, mice were lightly anesthetized with Metofane inhalation and treated with 20 mg p.o. encapsulated enteric-coated OVA or saline microbeads, followed by 300 μ l acidified water (pH 2). Mice were subsequently analyzed 72 h after the last oral treatment.

Blood eosinophil analysis

Peripheral blood samples were collected in heparinized tubes (Becton Dickinson, Franklin Lakes, NJ) by tail bleeding. Blood eosinophil levels were determined by counting cells with Neubauer hemacytometer by staining whole blood with Discombe's solution (23).

Eosinophil analysis in the esophagus

The esophagus of adult mice was fixed in 4% paraformaldehyde in phosphate buffer, pH 7.4, embedded in paraffin, cut into 5- μ m sections, fixed to positive charged slides, and immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP), a kind gift of J. and N. Lee (Mayo Clinic, Scottsdale, AZ), as described (16, 24). In brief, endogenous peroxidase in the tissues was quenched with 0.3% hydrogen peroxide in methanol, followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rabbit anti-MBP (1/16,000) overnight at 4°C, followed by 1/200 dilution of biotinylated goat anti-rabbit IgG secondary Ab and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each. These slides were further developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate, and counterstained with nuclear fast red. Negative controls include replacing the primary Ab with normal rabbit serum to check endogenous biotin and peroxidase activity. Quantification of immunoreactive cells was conducted by counting the positive stained cells on each tissue section using a 10 \times 10- μ m ocular micrometer (B & B Microscopes, Warrendale, PA), and eosinophil levels are expressed as cells/mm².

ELISA measurements

The level of murine IL-5 was determined as previously reported (22). The serum level of IgE was determined using the OPTeIA mouse IgE assay (BD Pharmingen, San Diego, CA). The serum level of human IL-5 4 days after the placement of a saline or IL-5-secreting miniosmotic pump was determined using the Quantikine human IL-5 assay (R&D Systems, Minneapolis, MN).

Statistical analysis

Statistical significance comparing different sets of mice was determined by Student's *t* test. A value of *p* < 0.05 was considered statistically significant.

Results

Transgenic overexpression of IL-5 with a T cell promoter induces esophageal eosinophilia

We were interested in dissecting the mechanism involved in regulating esophageal eosinophilia. As an initial analysis, we inves-

tigated eosinophil levels in the esophagus of mice transgenic for IL-5 under the control of the T cell promoter CD2. These transgenic mice were found to have >50-fold increase in the number of esophageal eosinophils compared with wild-type mice (Fig. 1). As a control, the number of eosinophils in the small intestine of IL-5-transgenic and wild-type control mice was measured in parallel and exhibited a smaller increase in eosinophils (only \sim 5-fold higher) (Fig. 1). For example, the numbers of eosinophils in the esophagus of wild-type and IL-5-transgenic mice were 1.92 ± 0.9 and 121 ± 14 eosinophils/mm² (mean \pm SEM, *n* = 10), respectively, in comparison with the small intestine, in which the numbers were 44 ± 5 and 193 ± 18 eosinophils/mm² (mean \pm SEM, *n* = 10), respectively. Eosinophils in IL-5-transgenic mice were distributed in each segment of the esophagus from external layers of the loose connective tissue, the muscularis region, and submucosa (Fig. 2). Occasionally, intraepithelial eosinophils were also seen (Fig. 2D); however, no disruption of the integrity of the epithelial cell layer was observed in the IL-5-transgenic mice.

Intestinal IL-5-transgenic mice have elevated esophageal eosinophils

We were next interested in determining whether transgenic overexpression of IL-5 under the regulation of another promoter also promoted esophageal eosinophilia. To test this hypothesis, we examined mice that overexpressed IL-5 under the control of the rat fabpi intestine promoter. This promoter is specifically expressed in the small intestine (19, 21, 25). Mice overexpressing intestinal IL-5 had 14.5 ± 3.2 eosinophils/mm² (mean \pm SEM, *n* = 8) in comparison with wild-type littermate control mice, which had 1.26 ± 0.85 eosinophils/mm² (mean \pm SEM, *n* = 8, *p* < 0.001). For comparison, we also examined intestinal eotaxin-transgenic mice. Interestingly, eotaxin-transgenic mice had esophageal eosinophil levels similar to wild-type mice. The eosinophil levels in intestinal eotaxin-transgenic mice were 1.73 ± 1 eosinophils/mm² (mean \pm SEM, *n* = 8) (Fig. 3). It is relevant to note that intestinal IL-5 and eotaxin-transgenic mice have intestinal eosinophilia; however, only intestine IL-5-transgenic mice have blood eosinophilia (21). Likewise, intestine IL-5-transgenic mice have elevated levels of serum IL-5 compared with wild-type mice (45.6 ± 12 and 69.4 ± 12.9 ng/ml (mean \pm SD, *n* = 4; *p* < 0.05) for wild-type and IL-5-transgenic mice, respectively), whereas eotaxin-transgenic mice have normal serum levels of IL-5 (50.7 ± 14 ng/ml). In addition to having elevated levels of circulating and intestinal eosinophils, intestine IL-5-transgenic mice have elevated levels of

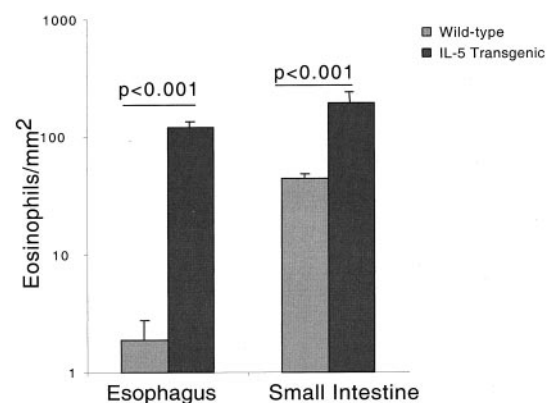


FIGURE 1. Effect of the CD2-IL-5 transgene on eosinophil levels in the esophagus and small intestine. The level of eosinophils in the esophagus and small intestine of wild-type and CD2-IL-5-transgenic mice is shown. The results are expressed as mean \pm SEM, *n* = 10 mice.

³ Abbreviations used in this paper: fabpi, fatty acid-binding protein; MBP, major basic protein.

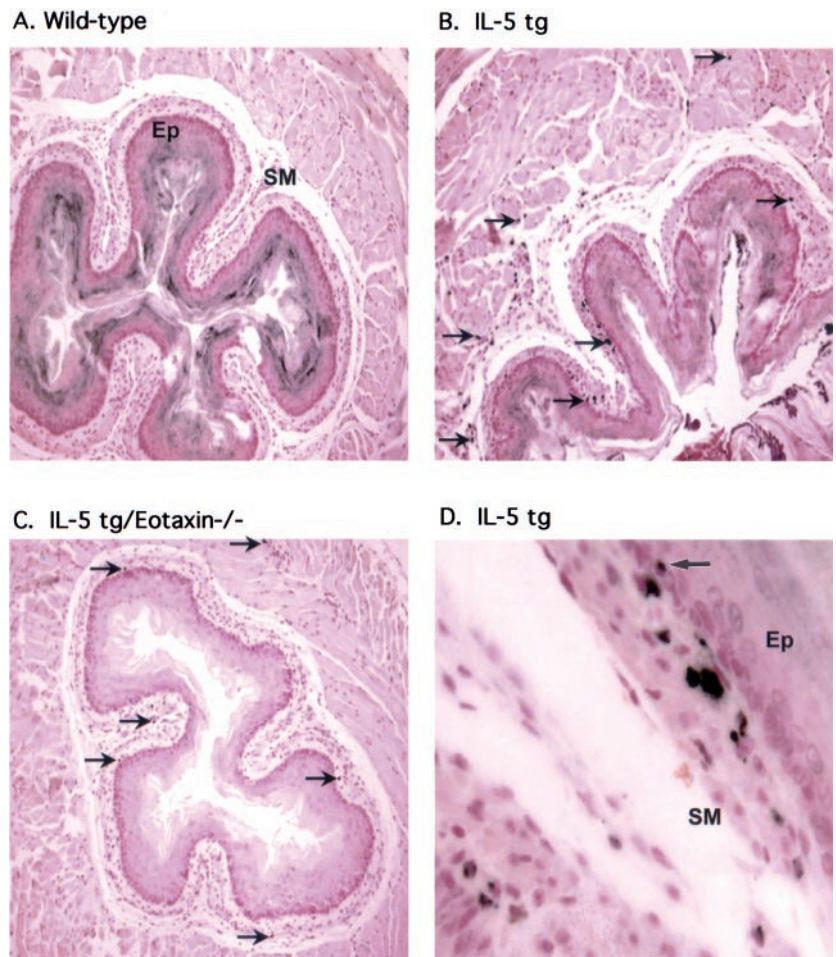


FIGURE 2. Immunohistochemical detection of eosinophils. Eosinophils were identified by anti-MBP staining, and representative cells are noted by arrows. Representative sections from wild-type (A), CD2-IL-5-transgenic (B and D), and CD2-IL-5-transgenic eotaxin-deficient mice (IL-5 Tg/eotaxin^{-/-}) (C) are shown. D, A high power magnification depicts eosinophils in proximity to the epithelial layer; a representative intraepithelial eosinophil is noted with the arrow. Magnification is $\times 100$ (A), $\times 125$ (B and C), and $\times 400$ (D). Ep, epithelium; SM, submucosa.

IgE (291 ± 144 and 2730 ± 1350 ng/ml (mean \pm SD, $n = 4$; $p < 0.001$) for wild-type and transgenic mice, respectively).

Pharmacological administration of systemic IL-5 promotes recruitment of eosinophils to the esophagus

We were next interested in determining whether elevated systemic levels of IL-5 promoted eosinophil trafficking to the esophagus. We tested this hypothesis by pharmacological administration of IL-5 via a miniosmotic pump placed in the peritoneal cavity. IL-5

administration resulted in marked elevations of eosinophils in blood and esophagus compared with mice treated with saline alone (Fig. 4). For example, the number of eosinophils in the blood increased from $8 \pm 1 \times 10^4$ to $93 \pm 11 \times 10^4$ cells/ml (mean \pm SEM, $n = 6$) and $108 \pm 14 \times 10^4$ cells/ml following 500 pmol and 1000 pmol/kg IL-5, respectively. In the esophagus, eosinophil levels increased from 1.1 ± 0.7 eosinophils/mm² to 11.4 ± 3.4 and 24.8 ± 3.7 eosinophils/mm² (mean \pm SEM, $n = 6$) following 500 and 1000 pmol/kg IL-5, respectively. Consistent with the miniosmotic pump elevating systemic levels of IL-5, the serum human

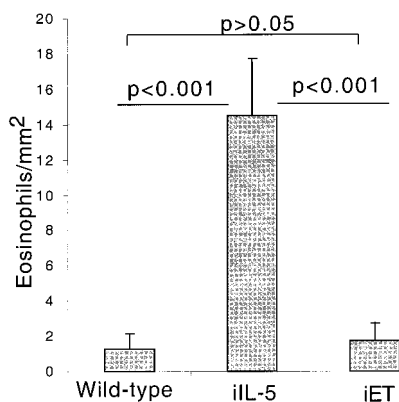


FIGURE 3. Esophageal eosinophil levels in IL-5 and eotaxin intestine-transgenic mice. Eosinophil levels in the esophagus of wild-type, IL-5 intestine-transgenic (iIL-5) mice, and eotaxin intestine-transgenic (iET) mice were determined by morphometric analysis following anti-MBP staining. Results are expressed as mean \pm SEM, $n = 8$ mice.

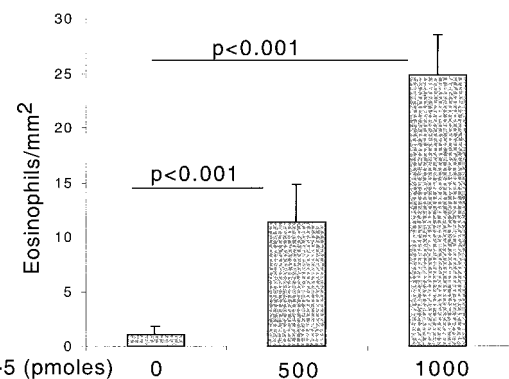


FIGURE 4. Effect of systemic elevations of IL-5 on esophageal eosinophil levels. IL-5 was delivered to mice by systemic administration through a miniosmotic pump placed in the peritoneal cavity. The eosinophil levels at two concentrations of IL-5 are shown after 8 days and are expressed as mean \pm SEM ($n = 6$ in each group).

IL-5 level was 15.3 ± 7.3 and 223 ± 21 ng/ml (mean \pm SD, $n = 4$, $p < 0.001$) 4 days after insertion of the control or IL-5 (4 pmol/kg/h)-secreting miniosmotic pumps, respectively. IL-5 delivery via the miniosmotic pump did not affect serum IgE levels (317 ± 179 and 311 ± 100 ng/ml for control and IL-5-secreting pumps, respectively). Collectively, these results establish that systemic elevations in IL-5 are sufficient for eosinophil trafficking to the esophagus.

Eosinophil trafficking to the esophagus following oral Ag challenge is IL-5 dependent

We were next interested in establishing whether IL-5 had an obligatory role in promoting esophageal eosinophils during the induction of experimental eosinophilic esophagitis associated with eosinophilic gastroenteritis. We therefore subjected IL-5-deficient and wild-type mice to the oral allergen challenge under conditions that promote eosinophil-associated gastrointestinal inflammation involving the esophagus, stomach, and intestine (26). We chose to focus on the 72-h time point after the allergen challenge, since this is when there is maximum Ag-induced esophageal eosinophilia. For example, in a kinetic analysis 6, 24, 48, and 72 h after placebo and allergen challenge, a significant difference between placebo and OVA-induced esophageal eosinophils was only seen at the 48- and 72-h time points; esophageal eosinophils were 27 ± 8.4 vs 53 ± 14 (mean \pm SD, $n = 4$, $p < 0.05$) and 11 ± 5.3 vs 45 ± 8.4 ($p < 0.001$) for placebo and OVA-challenged mice at 48 and 72 h, respectively (and data not shown). IL-5-deficient mice did not mount Ag-induced esophageal eosinophilia following OVA challenge. In contrast, following OVA challenge, sensitized wild-type mice had an increase in esophageal eosinophil levels in comparison with saline-challenged mice (Fig. 5). The eosinophil numbers in the esophagus of challenged wild-type mice were 28.2 ± 4.3 in comparison with 7.3 ± 0.9 eosinophils/mm² (mean \pm SEM, $n = 6$, $p < 0.001$) in saline-challenged wild-type mice, whereas IL-5-deficient mice had 12.3 ± 3.2 and 9.5 ± 1.7 eosinophils/mm² (mean \pm SEM, $n = 6$) following saline and OVA challenge, respectively.

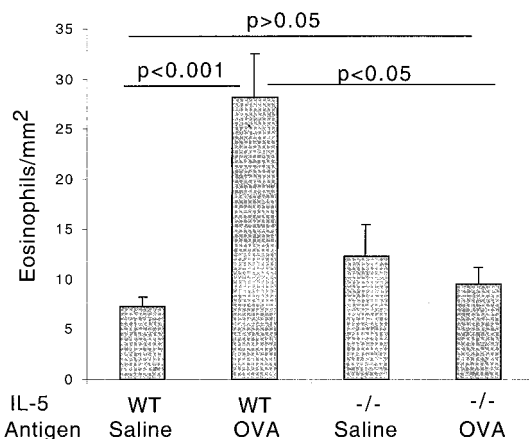


FIGURE 5. Role of IL-5 in oral Ag-induced eosinophil trafficking to the esophagus. OVA-sensitized IL-5 gene-targeted mice and strain-matched wild-type (WT) controls were challenged with enteric-coated OVA or saline placebo beads. The number of eosinophils in the esophagus was determined by anti-MBP staining and is expressed as mean \pm SEM ($n = 8$ in each group). The analysis is performed at 72 h after the allergen challenge since this is the maximum time point for eosinophil recruitment to the gastrointestinal tract (22, 26).

IL-5-mediated eosinophil recruitment to the esophagus is partially dependent on eotaxin

We were next interested in determining the relationship between IL-5 and eotaxin in regulating eosinophil levels in the esophagus. To address this, we generated CD2-IL-5-transgenic mice that were genetically wild type, heterozygote, or deficient in eotaxin, and evaluated the presence of eosinophils in the esophagus. The level of eosinophils in the esophagus was markedly increased in IL-5-transgenic mice and respectively reduced in eotaxin heterozygote- and homozygote-deficient mice (Fig. 6). In the absence of eotaxin, there was a 15-fold reduction in the number of eosinophils in the esophagus compared with IL-5-transgenic mice (Fig. 2C). For example, the numbers of eosinophils in the esophagus of IL-5-transgenic mice were 121 ± 14 eosinophils/mm² (mean \pm SEM, $n = 8$), whereas, in eotaxin-deficient IL-5-transgenic heterozygote and homozygote mice, their numbers were reduced to 22.3 ± 1.3 and 7.8 ± 1.7 eosinophils/mm² (mean \pm SEM, $n = 9$), respectively. It is interesting to note that in the absence of eotaxin, the IL-5-transgenic mice still had more eosinophils compared with wild-type mice (Fig. 6). Thus, eotaxin is partially required for IL-5-mediated eosinophil trafficking to the esophagus.

Discussion

Eosinophil infiltration into the esophagus is a commonly observed medical problem in patients with diverse diseases including gastroesophageal reflux, drug reactions, allergic eosinophilic esophagitis, eosinophilic gastroenteritis, and primary eosinophilic esophagitis (4–6, 27, 28). Recent clinical studies have suggested that the level of eosinophils in the esophagus negatively correlates with response to conventional gastroesophageal reflux therapy (29). Additionally, recent studies with aeroallergen-induced experimental eosinophilic esophagitis have established a causal role of esophageal eosinophils for the development of epithelial hyperplasia (10). These findings highlight the importance of dissecting the mechanism of eosinophil trafficking to the esophagus. In the current study, we elucidate several principles concerning the mechanism of eosinophil trafficking to the esophagus. We demonstrate that overproduction of IL-5 in vivo, by transgenic regulation with a T cell promoter (CD2), induces eosinophil accumulation in the esophagus. To determine whether this was mediated by local effects of IL-5 liberated from CD2⁺ T cells in the esophagus, we

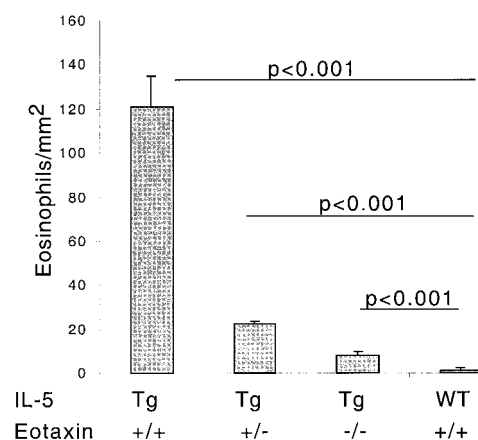


FIGURE 6. Cooperation of the IL-5 transgene and eotaxin in regulating esophageal eosinophil levels. The level of eosinophils in the esophagus of mice carrying the IL-5-CD2 transgene (Tg) with the wild-type (WT), heterozygote (+/-), or homozygote (-/-) deficiency of eotaxin. The eosinophil level in wild-type mice is also shown. Data are expressed as mean \pm SEM ($n = 8$ –9 for each group).

also examined transgenic mice that overproduced IL-5 under the control of a promoter not expressed by cells in the esophagus, the intestine-specific promoter, *fabpi* (19, 21, 25). These results revealed that IL-5 intestine-transgenic mice also had elevated eosinophil trafficking to the esophagus. Furthermore, we demonstrated that elevation of IL-5 levels via pharmacological administration also induced esophageal eosinophilia. Collectively, these results support a systemic mechanism for the action of IL-5. Indeed, CD2-IL-5-transgenic mice have elevated serum levels of IL-5 compared with wild-type mice (37 ± 11 vs <1 U/ml) (15). Additionally, oral allergen-challenged mice have elevated IL-5 production by splenocytes (22). Interestingly, eosinophil responses in the esophagus are similar to their responses in Peyer's patches, where elevated systemic levels of IL-5 also induce eosinophil accumulation (18). Eosinophil recruitment to the esophagus is also IL-5 dependent following exposure to aeroallergen extracts from *Aspergillus fumigatus* (10). However, systemic Th2 responses do not always lead to eosinophilic esophagitis since mice with experimental asthma induced by intranasal OVA (which also induces circulating eosinophilia) do not develop significant esophagitis (10). IL-5 is known to prime eosinophils to respond to chemoattractants and to induce eosinophil adhesion molecule expression and activation. Thus, IL-5 may induce eosinophil trafficking to the esophagus by enhancing eosinophil responsiveness to endogenous chemokines expressed by the esophagus, such as eotaxin or by up-regulating homing receptors specifically involved in eosinophil trafficking to the esophagus (30). Eotaxin is a constitutively expressed chemokine in the esophagus (16), and to evaluate its role in mediating IL-5-induced eosinophil trafficking to the esophagus, we examined CD2-IL-5-transgenic mice that were genetically deficient in eotaxin. These studies revealed that eotaxin had a significant role in IL-5-mediated esophageal eosinophilia, but they also demonstrated eotaxin-independent trafficking since IL-5-transgenic/eotaxin-deficient mice still had higher esophageal eosinophils than in wild-type mice. Consistent with this, eosinophils respond to a variety of chemokines including other CCR3 ligands (monocyte chemoattractant protein-2 and -3, RANTES, and eotaxin-2 and -3) (31–33); it remains to be determined which chemoattractants are responsible for mediating eotaxin-independent eosinophil accumulation in the esophagus.

In addition to demonstrating that IL-5 overexpression is sufficient for inducing eosinophil accumulation in the esophagus, we also demonstrate that IL-5 is required for oral Ag-induced eosinophil trafficking to the esophagus. In the absence of IL-5 (by analysis of gene-targeted mice), esophageal eosinophilia induced by exposure of OVA-sensitized mice to enteric-coated OVA beads is markedly reduced. This is significant because these oral Ag-challenged IL-5-deficient mice still mount intestinal eosinophilia (22). Thus, there is a differential requirement of IL-5 in regulating esophageal and intestinal eosinophilia. A clinical study has demonstrated elevated levels of IL-4-secreting T cells in esophageal lesions of patients with secondary eosinophilic esophagitis, supporting a central role of Th2 cell responses in the pathogenesis of eosinophilic esophagitis (34). The identification of a Th2-associated cytokine with the immunopathogenesis of eosinophilic esophagitis suggests that drugs used to treat allergy may also be useful for eosinophilic esophageal disorders. Interestingly, recent clinical studies have shown that topical delivery of glucocorticoids to the esophagus is effective therapy in some patients with eosinophilic esophagitis (7, 8, 35). Additionally, humanized anti-IL-5, a therapeutic reagent currently being studied for patients with asthma (36), may also be useful for the treatment of eosinophilic esophagitis.

In summary, these investigations dissect the cellular and molecular mechanisms involved in eosinophil homing to the esophagus. These data demonstrate that IL-5 overexpression by independent approaches induces eosinophil trafficking to the esophagus. Additionally, in an experimental model of oral Ag-induced eosinophilic gastroenteritis, IL-5 is demonstrated to have a critical role in regulating eosinophil trafficking to the esophagus. Taken together with the critical role of IL-5 in the induction of aeroallergen-induced eosinophilic esophagitis (10), these studies highlight the dominant role of IL-5 in regulating eosinophil accumulation in the esophagus.

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