

Targeted Disruption of the Chemokine Eotaxin Partially Reduces Antigen-induced Tissue Eosinophilia

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Summary

The chemokines are a large group of chemotactic cytokines that regulate leukocyte trafficking and have recently been shown to inhibit human immunodeficiency virus entry into cells. Eotaxin is a C-C chemokine implicated in the recruitment of eosinophils in a variety of inflammatory disorders and, unlike all other eosinophil chemoattractants, is eosinophil specific. However, given the large number of chemoattractants that have activities on eosinophils, it is unclear whether eotaxin has an important role in vivo. Furthermore, it remains unclear why there is constitutive expression of eotaxin in healthy states in the absence of eosinophilic inflammation. To begin to determine the significance of eotaxin at baseline and during eosinophil-mediated disease processes, we have used targeted gene disruption to generate mice that are deficient in eotaxin. Such mice demonstrate that eotaxin enhances the magnitude of the early (but not late) eosinophil recruitment after antigen challenge in models of asthma and stromal keratitis. Surprisingly, a role for eotaxin in regulating the constitutive number of eosinophils in the peripheral circulation is also demonstrated. These results indicate a contributory role for eotaxin in the generation of peripheral blood and antigen-induced tissue eosinophilia.

Eosinophils are potent pro inflammatory cells involved in the pathogenesis of several human disorders such as asthma and chronic parasitic infections (1). A better understanding and treatment of these diseases involves elucidating the mechanisms that regulate the selective accumulation of eosinophils. The generation of tissue eosinophilia involves multiple processes, including eosinophil hematopoietic development, endothelial adhesion, chemotaxis, and survival. Whereas eosinophil growth factors, such as IL-5, are involved in eosinophil hematopoiesis and survival, adhesion and locomotion are predominantly controlled by chemoattractants.

The chemokines are a super family of chemotactic cytokines that mediate leukocyte trafficking by binding to specific G protein linked seven transmembrane spanning receptors (2). Recently, chemokine receptors have been identified as coreceptors for HIV entry into cells and chemokines have been shown to be inhibitors of HIV replication (3). Chemokines are divided into three groups based on the primary sequence of the first two cysteines: the C-X-C, C-C, and C families. Whereas the C-X-C and C families are mainly active towards neutrophils and lymphocytes, respectively, the C-C family members are active towards macrophages, lymphocytes, basophils, and eosinophils. The eosinophil active chemokines include RANTES, macro-

phage chemotactic protein (MCP)-2, MCP-3, MCP-4, macrophage inflammatory protein (MIP)-1 α , and eotaxin (4). Eotaxin is the chief eosinophil chemotactic activity released into the lung in a guinea pig model of eosinophilic airway hypersensitivity (5) and its mRNA is induced in multiple animal models of eosinophilic inflammation and in human tissue in response to allergen challenge (6-12). When eotaxin is delivered to experimental animals in vivo, it induces a potent and rapid eosinophil-specific recruitment that is augmented by IL-5 (13, 14). However, eotaxin may have other activities given that its mRNA is constitutively expressed at high levels in multiple tissues in the absence of eosinophilic inflammation (6-8, 10), it has weak macrophage chemoattractive activity at high doses in vitro (8), its receptor is expressed at low levels in other activated leukocytes (15), and eotaxin can inhibit the replication of certain HIV strains in vitro (16). However, the role of eotaxin during eosinophil-mediated disease states is not known. If the activity of eotaxin is indeed restricted to eosinophils, then interference with its function would have clinical utility provided that the other chemoattractants do not have redundant activity. Using a gene disruption strategy, two other chemokines have been demonstrated to have nonredundant roles: MIP-1 α in the pathogenesis of viral induced inflammation (17) and stromal cell-derived factor-1 in B cell

lymphopoiesis and myelopoiesis (18). To explore further the function of eotaxin, we have used gene targeting to create mice with a genetic deficiency in eotaxin.

Materials and Methods

Targeting and Generation of Eotaxin-disrupted Mice. An eotaxin-targeting construct (Fig. 1 A) was constructed by cloning a 5' fragment containing a 5.0-kb NotI-XhoI fragment and a 3' fragment containing a 3.0-kb EcoRV-Xba I fragment into a vector (pPNT) that contained a neomycin-resistant gene (PGK-*neo*) and a herpes simplex virus thymidine kinase (*HSV-TK*) gene in tandem. The vector was linearized and transfected into TC1 embryonic stem cells (19) and 10^5 resistant clones were screened by Southern blot analysis using a 1.4-kb StuI-XbaI probe (Fig. 1 A); four positive clones were detected. Two targeted clones were injected into C57BL/6J blastocysts by standard methods. Both clones produced high degree chimeras, which transmitted the disrupted eotaxin allele to offspring at high frequency, allowing this allele to be established in a mixed background (129SvEv \times NIH Black Swiss Webster). Siblings from mixed heterozygote matings were used to control for strain background effects. All mice were housed in a germ-free barrier facility.

Genotyping was performed by Southern blot analysis of tail DNA using the 1.4-kb probe described above. For Northern analysis, total RNA was isolated using Trizol Reagent (GIBCO BRL, Gaithersburg, MD) and 20 mcg was electrophoresed into agarose, transferred to Gene Screen (Dupont-New England Nuclear, Boston, MA), and hybridized with a full-length murine eotaxin cDNA or a 28S rRNA cDNA (7).

Hematological Analysis. Tail or retro orbital vein blood samples were placed into EDTA containing tubes and subjected to automated cell counting. Peripheral smears were stained with Wright-Giemsa and analyzed at $1,000\times$ for the cell differential by an observer unaware of each genotype. For flow cytometric analysis, splenocytes or thymocytes were stained with antibodies and subjected to flow cytometry in a Cytofluorograph II flow cytometry machine (Ortho Diagnostic Systems, Inc., Raritan, NJ). The FITC-conjugated monoclonal antibodies were the following: rat anti-mouse B220, CD3, CD5, CD8, CD18, CD23, CD24, T200, and Gr-1; PE-conjugated monoclonal antibodies were rat anti-mouse T cell receptor $\gamma\delta$ and CD4; FITC-conjugated goat polyclonal antiserum were anti-mouse IgM and Ig κ ; and FITC-hamster anti-mouse was anti-T cell receptor $\alpha\beta$. Additionally, unlabeled primary mAbs were rat anti-mouse Mac-1, FcR (2.4G2), PgP-1, Mel 14, and VLA-4, which were used with secondary FITC-labeled goat anti-rat IgG.

Experimental Allergic Airway Disease. The airway sensitization protocol has been previously described (20). Mice were immunized intraperitoneally with 10 mcg of OVA (Sigma Chemical Co., St. Louis, MO) and 1 mg aluminum hydroxide intraperitoneally on days 0, 7, and 14. Sham-immunized mice received aluminum hydroxide alone. Mice underwent aerosol challenge with OVA (50 mg/ml in sterile saline) 7–10 d after the final immunization. Bronchoalveolar lavage (BAL) analysis was performed at either 18 or 48 h after aerosol challenge.

Experimental Onchocercal Keratitis. Mice were sensitized to soluble parasite antigens (OvAg) by 4 weekly s.c. immunizations with 10 mcg OvAg together with STP adjuvant as previously described (21). For intracorneal challenges, 1 wk after the final sensitization, 10 mcg OvAg was injected into the corneal stroma of the right eye; in some experiments, OvAg was injected in the contralateral corneas 1 d before killing, thereby providing tissue

from day 1 and 8 after challenge. Rabbit antisera to murine eosinophil major basic protein was a gift of Dr. G. Gleich of the Mayo Clinic (Rochester, MN) and used to detect tissue eosinophils. Statistical differences between groups were determined by the Student's *t* test.

Thioglycollate-induced Peritoneal Inflammation. Mice were injected intraperitoneally with 1.0 ml of 2.9% (wt/vol) thioglycollate and the peritoneal cells analyzed after 48 h as previously described (7).

Results and Discussion

Generation of Eotaxin Null Mice. The eotaxin gene is composed of three exons: exon 1 encodes the leader sequence, exon 2 encodes most of the mature protein, and exon 3 encodes predominantly untranslated mRNA (7). To disrupt the eotaxin gene, a targeting strategy was employed that deleted 1.4 kb of DNA corresponding to all of exon 2 and most of exon 3 (Fig. 1 A) and thus producing a null mutation. Clones that underwent homologous recombination were screened by Southern blot analysis using the 1.4-kb 3' probe. Two targeted clones were injected into blastocysts, and both transmitted the disrupted eotaxin allele in the germline of mice. Animals with a mixed background were generated by mating chimeras with NIH Black Swiss Webster mice. No phenotypic variance was detected between the two different knockout lines.

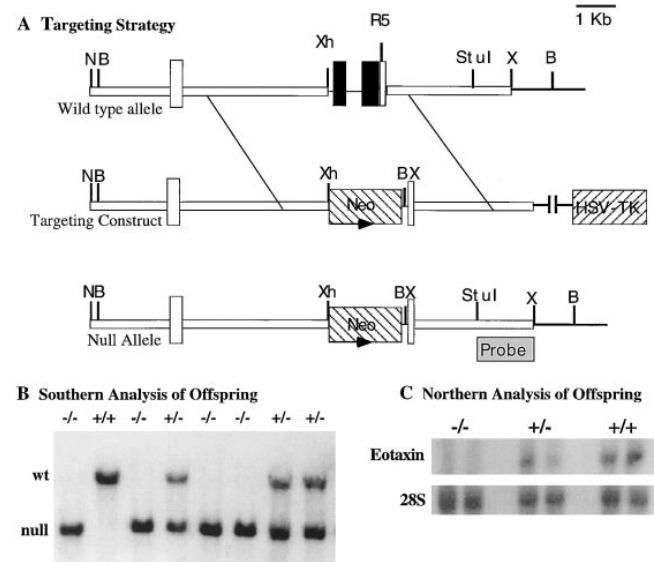


Figure 1. Generation of eotaxin-disrupted mice. Shown in (A) is the eotaxin genomic locus, the targeting vector, and the targeted null locus. Vertical rectangles represent exons; black regions in the exons are deleted by the targeting strategy. The targeting vector contains a neomycin-resistant gene (*neo*) and a herpes simplex virus thymidine kinase (*HSV-TK*) gene in tandem. In (B), Southern analysis of BamHI-digested genomic DNA from the offspring of a heterozygous cross. Wt and null indicate wild-type and targeted locus, respectively. (C) shows a Northern analysis of skin total RNA (20 mcg) from homozygote null ($-/-$), heterozygote ($+/-$), and wild-type mice ($+/+$). The Northern blot was hybridized with a full-length murine eotaxin cDNA or 28S rRNA probe. Restriction enzyme abbreviations: N, NotI; B, BamHI; Xh, XhoI; R5, EcoRV; X, XbaI.

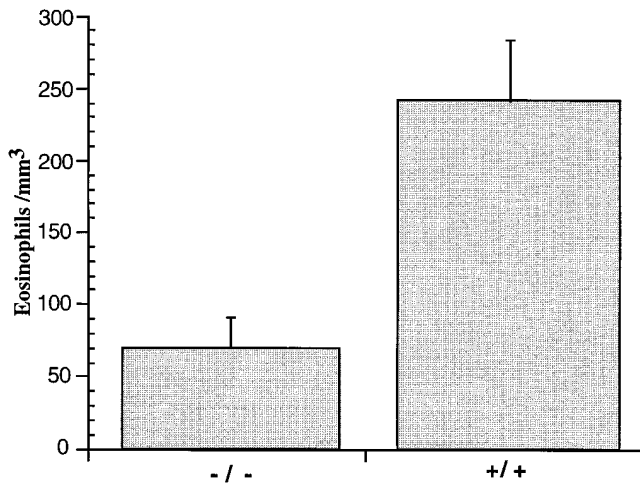


Figure 2. Eosinophil count in the peripheral blood of naive mice. The absolute eosinophil count in the peripheral blood is shown for wild-type (+/+) and eotaxin null (-/-) mice. The results are expressed as mean \pm SEM for +/+ ($n = 12$) and -/- ($n = 14$); $P = 0.007$.

F1 heterozygous offspring were intercrossed, and F2 offspring were genotyped by Southern blot analysis. All three genotypes were detected in F2 litters (Fig. 1 B). Cumulative genotyping of heterozygous crosses from mixed background matings revealed that the ratios of wild-type/heterozygote/homozygote mutant mice were 45:91:55. These ratios were not different from the expected 1:2:1 Mendelian ratios, indicating that the eotaxin gene disruption did not affect viability. This is in contrast with the gene targeting of another chemokine stromal cell-derived factor-1, which resulted in perinatal lethality (18).

The mouse skin is normally a rich source of eotaxin mRNA constitutively. To verify that the eotaxin targeting strategy had generated a true eotaxin gene disruption, eotaxin mRNA was examined by Northern blot analysis of skin RNA. Wild-type and heterozygote mice had mRNA of the predicted size; in contrast, eotaxin-targeted mice had no detectable eotaxin mRNA (Fig. 1 C). This indicated that the targeting strategy had indeed generated eotaxin null mice.

Hematological and Lymphoid Analysis. One of the characteristic features of eotaxin that remains puzzling is its widespread (although not ubiquitous) constitutive expression in various tissues, especially lymphoid tissue (thymus and lymph node). It has been postulated that constitutive eotaxin might affect eosinophil and/or lymphocyte homing into these tissues (6). Therefore it was of interest to examine various organs and their resident leukocyte populations. Peripheral blood analysis revealed no abnormalities in the total leukocyte, red blood cell, and platelet counts, or white blood cell differential in eotaxin null mice compared with wild type mice (data not shown). In contrast, the total eosinophil count was significantly reduced ($P = 0.007$) in the knockout mice compared with wild-type mice (Fig. 2). Wild-type mice and null mice had 243 ± 43 (mean \pm SEM, $n = 12$) and 69 ± 22 ($n = 13$) eosinophils/mm³ of blood, re-

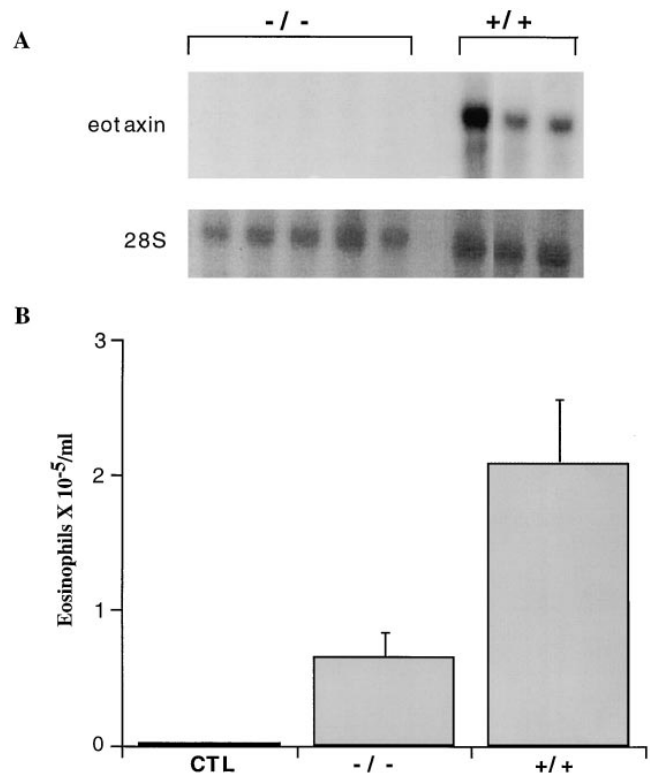


Figure 3. Allergen induced airway eosinophilia. Mice were sensitized systemically to OVA and underwent inhaled OVA challenge. At 18 h after allergen challenge, the lungs were assessed for eotaxin mRNA expression (A) and the eosinophil count in the bronchoalveolar fluid (B). Northern blot analysis of total RNA evaluating eotaxin expression in eotaxin null (-/-) and wild-type mice (+/+). The hybridization of a 28S rRNA cDNA probe is also shown. Each lane represents a different mouse. In (B), the number of eosinophils in the lung fluid is shown as mean \pm SEM for wild-type ($n = 10$), eotaxin null ($n = 13$), or control unsensitized mice ($n = 3$); $P = 0.005$ between +/+ and -/-.

spectively. Analysis of bone marrow cells revealed no differences in the eosinophil lineage (data not shown). This indicated that eotaxin was unlikely to be stimulating eosinophil hematopoiesis as might have been expected based on the ability of other chemokines (e.g., MIP-1 α , IL-8) to regulate hematopoiesis (22).

No gross or histological abnormalities were detectable in any organ, including those with abundant eotaxin expression (skin, thymus, heart, and intestine) (7). The leukocytes from the thymus and spleen from four wild-type and eotaxin null mice were subjected to flow cytometry analysis for cell surface and activation markers (data not shown). No abnormalities were seen in leukocyte phenotype using lymphocyte markers that included B220, Thy1, CD3, CD4, CD8, and CD23; additionally, no abnormalities were detected in adhesion molecules including CD18, VLA-4, or Pgp-1. Thus, the function of constitutive eotaxin does not appear to affect the number or phenotype of thymocytes or splenocytes. The only apparent effect of eotaxin deficiency was therefore on the baseline number of eosinophils in the peripheral blood.

Eosinophil Recruitment into Allergic Airways. Eotaxin has

been implicated in the recruitment of eosinophils into the lungs following allergen challenge (5, 6, 11). Therefore, it was important also to determine whether the deletion of eotaxin affected the recruitment of eosinophils into the lung during experimental allergic airway disease. When mice are sensitized to intraperitoneal OVA and then challenged with inhaled OVA, eotaxin lung mRNA is known to be induced rapidly (with peak expression at 3–6 h) and is accompanied by the development of an eosinophil-dependent allergic airway disease (6, 11, 20). At 18 h after allergen challenge, eotaxin mRNA was readily detectable in the lungs of wild-type mice, but not detectable in eotaxin null mice (Fig. 3 A). This confirmed that the eotaxin gene was indeed inactivated. At 18 h after the allergen challenge, the number of cells in the BAL fluid was assessed. No significant differences in the lymphocyte, neutrophil, or macrophage cell counts were detectable (data not shown). However, eosinophil numbers were reduced by 70% in the eotaxin null mice compared with identically treated wild-type mice ($6.6 \pm 1.7 \times 10^4$ versus $2.1 \pm 0.5 \times 10^5$, respectively, $P = 0.005$) (Fig. 3 B). This observation demonstrated that eotaxin was important in the early recruitment of eosinophils to the lungs in this model.

It is noteworthy that eotaxin null mice still mounted an eosinophil response compared with unsensitized control mice (Fig. 3 B), indicating the importance of other chemoattractants. The production of chemoattractants during an inflammatory response is a complicated dynamic process. Whereas resident epithelial cells appear to be the primary source of eotaxin and other chemokines at baseline and during the early part of the late phase inflammatory response, infiltrating cells including macrophages and eosinophils can also produce significant amounts of chemokines (10, 23). Therefore, we were interested in determining the role of eotaxin at a later time in the inflammatory response. At 48 h after antigen challenge, there was no longer a reduced number of eosinophils in the BAL fluid of eotaxin null mice compared with wild-type mice (data not shown). The role of eotaxin in this model appears to coincide with the peak induction of eotaxin mRNA at 3–6 h after antigen challenge (6, 11, 20).

Eosinophil Recruitment into Inflamed Corneas. Eosinophils also accumulate in other tissues besides the lungs, particularly during parasitic infections. Therefore, it was of interest to determine whether a role for eotaxin could be demonstrated in other inflammatory models. Inflammation of the corneal stroma (stromal keratitis) is a serious complication of infection with the nematode parasite *Onchocerca volvulus* and is a major cause of blindness (River Blindness) in Africa and Latin America. After sensitization with parasite antigens in mice, intracorneal antigen challenge induces pronounced corneal inflammation associated with infiltration of the cornea by eosinophils (21). The severity of corneal pathology is correlated with the local expression of chemokines including eotaxin (12). Wild-type and eotaxin null mice were immunized s.c. and then injected intracorneally with *O. volvulus* antigens. No difference was noted in the number of total cells in the cornea of wild-type and eo-

taxin null mice 1 d after antigen injection (mean \pm SEM/corneal section was 870 ± 90 versus 865 ± 44 , respectively; $P = 0.97$). In contrast, the number of eosinophils in the corneal stroma of eotaxin null mice was significantly less than in wild-type mice (105 ± 21 versus 204 ± 36 eosinophils/section; $P = 0.02$) (Fig. 4). To determine whether this reduction persisted at later times after antigen challenge, the eosinophil recruitment 8 d after *O. volvulus* antigen challenge was examined. Both groups of mice now had comparable numbers of eosinophils. Eosinophil numbers were 340 ± 60 and 440 ± 55 ($P = 0.26$) for wild-type and eotaxin null mice, respectively. These data indicate that eotaxin is important in early recruitment of eosinophils to the cornea during experimental helminth-mediated keratitis.

Cell Recruitment into the Peritoneal Cavity in Response to a Nonspecific Inflammatory Trigger. Because all C–C chemokines described have activities for macrophages and eotaxin has weak macrophage chemotactic activity in vitro (8), it was important to examine further the specificity of eotaxin in a model of inflammation known to induce macrophage recruitment. Inflammation in the peritoneal cavity was induced by the injection of a nonspecific inflammatory trigger, thioglycollate. At 48 h after injection, cells recovered from the peritoneal lavage were composed of 90% macrophages in both wild-type and knockout mice with the recovery of $1.1 \pm 0.1 \times 10^7$ and $1.2 \pm 0.05 \times 10^7$ macrophages/mouse (mean \pm SEM, $n = 5$), respectively, indicating that eotaxin had no role in macrophage recruitment in this model. There were no differences in the other 10% of the leukocytes recruited (data not shown).

The generation of eotaxin null mice has revealed several important biological properties of eotaxin. First, a definitive role for eotaxin in enhancing the magnitude of early eosinophil tissue recruitment after antigen challenge via

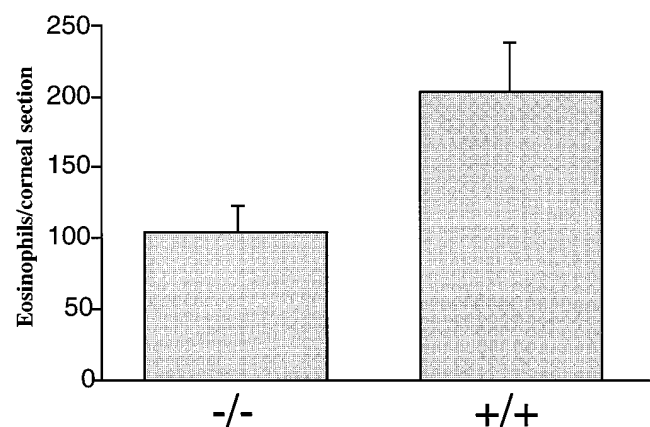


Figure 4. Antigen-induced corneal eosinophilia. Wild-type and eotaxin null mice received four subcutaneous immunizations with antigens from the parasitic helminth *O. volvulus*. Animals were subsequently injected intracorneally with parasite antigen and sacrificed 1 d later. 5- μ m sections of the cornea were immunostained with rabbit sera to eosinophil major basic protein and eosinophils in the corneal stroma were counted. Data are shown as mean \pm SEM for wild-type ($n = 13$) and eotaxin null ($n = 14$) mice with differences between groups being significant ($P = 0.02$).

two different routes of antigen exposure (mucosal and epithelial) is demonstrated. These two systems are likely to involve the generation of a large number of chemoattractants and to use diverse types of antigen-presenting cells. It should be pointed out that even in the absence of eotaxin, there is still abundant antigen-induced tissue eosinophilia, indicating an important role for other eosinophil chemoattractants. Eosinophils use two distinct chemokine receptors: C-C chemokine receptor-1 (CCR-1) and CCR-3 (9, 15, 24). Whereas CCR-3 is more highly restricted to eosinophils and binds eotaxin, RANTES, MCP-3, and MCP-4; CCR-1 is expressed by other leukocytes and binds MIP-1 α , MCP-3, and RANTES. Pharmacological blockade of eosinophilia therefore might interfere with chemokine receptor signal transduction in addition to blockade of eotaxin. In support of this, MIP-1 α has been shown to be partially responsible for eosinophil lung recruitment in response to a *Schistosoma mansoni* antigen (25). Additionally, other chemoattractants besides chemokines (e.g., LTB₄) appear to be in-

involved in eosinophil lung recruitment (26). Second, eotaxin is demonstrated to be eosinophil specific, because only eosinophils are reduced in the eotaxin null mice. Previous specificity studies were based on in vitro assays or administration studies in vivo. And third, eotaxin is found to exert an effect on the baseline level of eosinophils in the peripheral circulation. This effect does not appear to be mediated at the level of the bone marrow, suggesting that there is an alteration in eosinophil trafficking in the absence of eotaxin. While the mechanism of eosinophil growth and development has been extensively studied and found to predominantly depend upon IL-5 (27), little is understood concerning the involvement of eotaxin in the generation of circulating eosinophils. It is noteworthy that IL-5 null mice still have residual eosinophils, indicating the importance of other pathways in the generation of eosinophilia (27). Eotaxin gene-targeted mice should provide a useful genetic model to understand further the mechanism of baseline and antigen-induced eosinophilia.

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