

The Eotaxin Chemokines and CCR3 Are Fundamental Regulators of Allergen-Induced Pulmonary Eosinophilia¹

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The eotaxin chemokines have been implicated in allergen-induced eosinophil responses in the lung. However, the individual and combined contribution of each of the individual eotaxins is not well defined. We aimed to examine the consequences of genetically ablating eotaxin-1 or eotaxin-2 alone, eotaxin-1 and eotaxin-2 together, and CCR3. Mice carrying targeted deletions of these individual or combined genes were subjected to an OVA-induced experimental asthma model. Analysis of airway (luminal) eosinophilia revealed a dominant role for eotaxin-2 and a synergistic reduction in eotaxin-1/2 double-deficient (DKO) and CCR3-deficient mice. Examination of pulmonary tissue eosinophilia revealed a modest role for individually ablated eotaxin-1 or eotaxin-2. However, eotaxin-1/2 DKO mice had a marked decrease in tissue eosinophilia approaching the low levels seen in CCR3-deficient mice. Notably, the organized accumulation of eosinophils in the peribronchial and perivascular regions of allergen-challenged wild-type mice was lost in eotaxin-1/2 DKO and CCR3-deficient mice. Mechanistic analysis revealed distinct expression of eotaxin-2 in bronchoalveolar lavage fluid cells consistent with macrophages. Taken together, these results provide definitive evidence for a fundamental role of the eotaxin/CCR3 pathway in eosinophil recruitment in experimental asthma. These results imply that successful blockade of Ag-induced pulmonary eosinophilia will require antagonism of multiple CCR3 ligands. *The Journal of Immunology*, 2005, 175: 5341–5350.

Allergic disease (asthma, rhinitis, atopic dermatitis, and food allergy) is a growing threat to public health with ~30% of the population of industrialized countries being afflicted. Although numerous inflammatory cells contribute to various aspects of disease pathogenesis, mounting evidence has shown that the localization and activation of eosinophils within the tissue may be an important factor in disease pathogenesis (1, 2). Eosinophils are thought to exert their effects by release of granule proteins. Indeed, levels of eosinophil granule proteins (e.g., major basic protein (MBP³)) often correlate with the severity of allergic disease (3).

Accordingly, elucidating the mechanism of eosinophil tissue recruitment is a topic of intense research focus (4). Eosinophil recruitment into inflammatory sites has been shown to involve a number of cytokines (most notably the Th2 cell products IL-4, IL-5, and IL-13) (5–7), adhesion molecules (e.g., β_1 , β_2 , and β_7 integrins) (8), chemokines (e.g., RANTES and the eotaxins (9), and other molecules (e.g., acidic mammalian chitinase) (10). Of the cytokines implicated in modulating leukocyte recruitment, only

IL-5 and the eotaxins selectively regulate eosinophil trafficking (11). 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 following allergen exposure (12). However, Ag-induced tissue eosinophilia can occur independent of IL-5, as demonstrated by residual tissue eosinophils in trials using anti-IL-5 in patients with asthma (13) and IL-5-deficient mice (14). Recent studies have also demonstrated an important role for the eotaxin subfamily of chemokines in eosinophil recruitment to the lung (9, 15–17).

Eotaxin was initially discovered using a biological assay in guinea pigs designed to identify the molecules responsible for allergen-induced eosinophil accumulation in the lungs (11). Subsequently, using genomic analyses, two additional chemokines have been identified in the human genome, which encode for CC chemokines with eosinophil-selective chemoattractant activity, and have hence been designated eotaxin-2 and eotaxin-3 (18, 19). Eotaxin-2 and eotaxin-3 are only distantly related to eotaxin-1 because they are only ~30% identical in sequence and are located on different chromosomes (19, 20). The specific activity of all eotaxins is mediated by the selective expression of the eotaxin receptor, CCR3, a seven-transmembrane spanning, G protein-coupled receptor, primarily expressed on eosinophils (21, 22), while noted on a subset of Th2 cells (23) and mast cells in humans (24), little is known about the expression of CCR3 on cells other than eosinophils in the mouse. Notably, the eotaxin chemokines cooperate with IL-5 in the induction of tissue eosinophilia. IL-5 increases the pool of eotaxin-responsive cells and primes eosinophils to respond to CCR3 ligands (12, 25). Furthermore, when given exogenously eotaxin-2 cooperates with IL-5 to induce substantial production of IL-13 in the lung (16). The finding that IL-4 and IL-13 are potent inducers of the eotaxin chemokines, by a STAT6-dependent pathway, has provided an integrated mechanism to explain the eosinophilia associated with Th2 responses (9). Interestingly, an elegant

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³ Abbreviations used in this paper: MBP, major basic protein; DKO, double deficient; KO, knockout; BALF, bronchoalveolar lavage fluid.

study has recently identified that eosinophil recruitment to the lung is dependent on STAT6 and a bone marrow-derived lung tissue resident non-T or B cell, but surprisingly, the specific cell and the chemoattractants responsible remain unknown (26).

Although a variety of approaches have been used to determine the biological role of the eotaxin chemokines, most studies have primarily focused on eotaxin-1. Using eotaxin-1 gene-deficient mice or neutralizing Abs, eotaxin-1 has been shown to have a contributory role in the temporal and regional distribution of eosinophils in the lung in several Ag-induced models of asthma (27). However, in all studies, a significant number of residual eosinophils was found when eotaxin-1 was neutralized, indicating the importance of additional regulatory molecules. Preliminary evidence exists that these residual eosinophils require CCR3, at least in part (30, 31). Recently, CCR3 gene-targeted mice have been developed, and two reports have been published concerning the induction of experimental asthma (30, 31). Using a standard experimental asthma model induced by systemic sensitization with OVA/alum followed by respiratory OVA challenge, only a modest reduction in lung eosinophils was found (31). However, when the same CCR3-deficient mouse line was subjected to experimental asthma induction by epicutaneous OVA sensitization, there was a marked deficiency of lung and bronchoalveolar lavage fluid eosinophils (30). It was proposed that these apparently conflicting results may be related to the sensitization protocol (30), but the reason for this apparent discrepancy remains unknown and its role in the residual eosinophilia seen in eotaxin-1 neutralization studies remains uncertain.

Despite numerous studies demonstrating the induction and correlation of eosinophils with each of the eotaxin chemokines in the human respiratory tract, there is a surprising paucity of information concerning the relative importance of each individual eotaxin chemokine, other CCR3 ligands, and additional non-CCR3-dependent pathways. Elucidating the individual and combined role of each of these molecules in regulating Ag-induced pulmonary eosinophilia is not just an academic question because there are numerous therapeutic compounds in development aimed at inhibiting each eotaxin and CCR3 by a variety of approaches (21, 32). Accordingly, in this article, we aimed to define the exact contribution of each eotaxin chemokine in a standard model of OVA-induced pulmonary inflammation. Because a functional murine homologue of human eotaxin-3 has not yet been characterized, we concentrated on eotaxin-1 and eotaxin-2. To definitively address this critical question, we took a gene targeting approach to generate mice deficient in either eotaxin-1 or eotaxin-2 alone, eotaxin-1 and eotaxin-2 together, and the eotaxin receptor CCR3 (generated by a distinct strategy).

Materials and Methods

Generation of eotaxin-2-deficient and eotaxin-1/2 double-deficient (DKO) mice

Eotaxin-2-deficient mice were engineered by standard molecular techniques as described previously (33). In brief, a targeting construct was designed to ablate eotaxin-2 expression by replacing the first two exons of eotaxin-2 with a neomycin resistance gene (see Fig. 1A). Mice were genotyped by Southern blot showing a wild-type band of 28.3 kb and an eotaxin-2-deficient band at 8.6 kb and by PCR (wild-type product = 2.4 kb and eotaxin-2-deficient product = 2.2 kb) as described previously (33). Eotaxin-1/2 DKO mice were generated by breeding eotaxin-2-deficient mice with eotaxin-1-deficient mice in the 129 SvEv background (27) and genotyping F2 offspring. Mice carrying the eotaxin-1 gene-targeted allele were identified by Southern blot analysis, as described previously (27). Experiments were performed on 6- to 8-wk-old mice, and controls consisted of 129 SvEv mice of similar age. All mice were maintained in a specific pathogen-free vivarium, and all experiments with mice have been

reviewed by the Institutional Animal Care and Use Committee and were conducted in accordance with all applicable regulations.

Generation of CCR3-deficient mice

CCR3-deficient mice were designed and developed by VelociGene technology (34). In brief, the CCR3 gene was replaced by a reporter-selection cassette, which consists of a β -galactosidase (LacZ) enzyme gene and a neomycin resistance gene (see Fig. 1B). The knockout (KO)/reporter construct was created by bacterial homologous recombination into a bacterial artificial chromosome encoding CCR3 and was constructed so that the LacZ gene is placed in frame with the second amino acid of CCR3. The construct deletes the 1074-bp coding region of CCR3 contained in exon 2 of the gene. The KO/reporter construct was electroporated into 129S1/Sv-derived ES cells, CJ7 clone (28, 35). Targeted clones were identified by Taqman screening, using two probes in the CCR3 gene as loss-of-allele probes (29). Chimeric mice were generated by microinjecting targeted ES clones into C57BL/6 embryos. Mice were backcrossed twice to C57BL/6 mice, and heterozygote backcrossed mice were crossed to create homozygous CCR3 gene-targeted mice. Mice were identified as heterozygotes and homozygotes by Taqman assay using probes for the *NEO* and *LacZ* genes and the CCR3 loss-of-allele probes. Mice were genotyped by PCR (common primer (P1) AAATTTGGAATTCCTTCCTTC, wild-type primer (P2) AACACCTTCGGCTCTTTTC), and the LacZ primer (P3) GTCTGTCCTAGCTTCCTCACTG producing a wild-type band of ~350 bp and a deficient band of ~450 bp. The absence of CCR3 in homozygous KO mice was confirmed by Northern blot and flow cytometric analysis using a fluorescein-labeled monoclonal rat anti-mCCR3 Ab (see Fig. 1D). Experiments on CCR3-deficient mice were performed on 6- to 8-wk-old mice and background-matched wild-type controls derived from littermates.

OVA-induced model of pulmonary inflammation

A mouse model of allergic lung disease was established as described previously (36). In brief, mice were sensitized by i.p. injection of 100 μ g of OVA and 1 mg of alum in 200 μ l of sterile physiologic saline on two occasions separated by 2 wk. Two weeks after the last sensitization, lightly anesthetized mice (isoflurane inhalation) received two intranasal administrations of 50 μ g of OVA or control saline 3 days apart. Mice were sacrificed between 18 and 20 h after the second intranasal challenge.

Bronchoalveolar lavage fluid (BALF) collection and analysis

The mice were euthanized by CO₂ inhalation. Immediately thereafter, a midline neck incision was made, and the trachea was cannulated. The lungs were lavaged three times with 1.0 ml of PBS containing 1% FCS and 0.5 mM EDTA. The recovered BALF was centrifuged at 400 $\times$ g for 5 min at 4°C and resuspended in 200 μ l of PBS containing 1% FCS and 0.5 mM EDTA. Lysis of RBC was conducted using RBC lysis buffer (Sigma-Aldrich) according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of 5×10^4 cells were stained with Giemsa-Diff-Quick (Dade Diagnostics), and differential cell counts were determined.

Eosinophil blood levels

Peripheral blood (5 μ l) was added directly to 45 μ l of Discombe's solution, and the eosinophils were counted with a hemacytometer as reported previously (37).

Bone marrow eosinophil CFU analysis

Eosinophil CFU analysis was performed as described previously (38). In brief, nonadherent low-density mononuclear cells (1×10^5) were cultured at 37°C and 5% CO₂ in 35 $\times$ 10-mm tissue culture dishes in 1.0 ml of Methocult, 20% FCS, and 5.0 ng/ml recombinant mouse IL-5.

Ab staining and flow cytometry

In some experiments, cells were isolated from CCR3-deficient and wild-type mice 30 min after i.v. injection with 100 pM/kg (2.5 μ g) of murine rIL-5 (R&D Systems) as described in Mold et al. (39). Flow cytometry was performed with isotype control Abs or fluorescein-labeled monoclonal rat anti-mCCR3 Ab (R&D Systems), anti-CD3 ϵ -FITC, anti-CD4-FITC, anti-CD8-PE, and anti-B220-PE Abs (BD Pharmingen).

ELISA measurements

The murine eotaxin-1 ELISA was used as described previously (36). The murine eotaxin-2 ELISA was a newly developed sandwich-type ELISA with custom and commercial antisera. Briefly, Immulon 4HBX microtiter plates (Dynex Technologies) were coated with polyclonal rabbit anti-murine-eotaxin-2. Purified rabbit serum was collected by Pocono Farms, after

several injections of murine eotaxin-2 protein (PeproTech) and Freund's adjuvant. Rabbit serum containing the anti-eotaxin-2 Ab was purified by ammonium sulfate precipitation and dialyzed against three changes of 100-fold excess volume of PBS. Nonspecific binding was blocked, with 10% BSA in PBS. Sample was then added to each well. Then the second Ab, anti-murine eotaxin-2 (anti-MPIF-2; R&D Systems), was added. A detection antibody, polyclonal donkey-anti-goat IgG-HRP conjugated Ab (Santa Cruz Biotechnology), was then added. BD OptEIA tetramethylbenzidine substrate (BD Pharmingen) was used and the OD was read at 450 nm. The least sensitive of three experiments for eotaxin-1 and eotaxin-2 ELISAs were ~150 and 300 pg/ml, respectively.

Quantification of tissue eosinophil and mast cell levels

Eosinophils in the tissue were differentially stained using an antiserum against murine MBP (anti-MBP) as described previously (40). In brief, endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol followed by nonspecific protein blocking with normal goat serum. Tissue sections were then treated with pepsin (Digest-All 3; Zymed Laboratories) 4 min at room temperature and then incubated with rabbit anti-murine MBP Ab (1:5000, a kind gift from J. Lee (Mayo Clinic, Scottsdale, AZ)) 1 h at room temperature, followed by 1/500 dilution of biotinylated goat anti-rabbit IgG secondary Ab and avidin-peroxidase complex (Vector Laboratories) for 30 min each. These 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 using the Image Pro Plus imaging software system (Media Cybernetics). Five-micrometer tissue sections were also stained for mucosal mast cells with chloroacetate esterase activity as described elsewhere (41) and lightly counterstained with methyl green. At least three random sections per mouse were analyzed. Quantification of stained cells per square millimeter of trachea was performed by morphometric analysis using the Image Pro Plus imaging software system (Media Cybernetics). At least four selections per mouse were analyzed. For the lung, the subepithelial peribronchial tissue regions associated with medium sized bronchioles were quantified. For the jejunum, the villus and lamina propria regions were quantified. Total cell numbers were counted relative to the total tissue area. Calculated eosinophil and mast cell levels are expressed as cells/mm².

In situ hybridization of mouse lung

In situ hybridization was performed as described previously (42). In brief, murine eotaxin-1 cDNA in pBluescript plasmid was linearized by restriction enzyme digest with *Xho*I or *Eco*RI sense and antisense probes were generated by T7 and T3 RNA polymerase, respectively (Riboprobe Gemini Core System II transcription kit; Promega). Murine eotaxin-2 cDNA in pBluescript plasmid was linearized by restriction enzyme digest with *Xho*I or *Eco*RI and antisense and sense probes were generated by T7 and T3 RNA polymerase, respectively. The radiolabeled (α S35-UTP) probes were hybridized and washed under high-stringency conditions. Expression of each chemokine by cells in the airway was quantitated by counting the number of positive cells (having >10 granules/cell) located in the airway of at least seven medium-sized bronchioles (lined with columnar epithelia) per lung section, which had at least one inflammatory cell present. Data are expressed as positive cells per airway $\pm$ SEM.

Northern blot densitometry analysis

Mice were sacrificed by CO₂ inhalation. Immediately after collection of BALF, the right lobe of the lung from each mouse was homogenized in TRIzol, and RNA was purified per manufacturer recommendations. RNA (12 μ g) was electrophoresed through 1.5% agarose, transferred to a hybridization membrane, and probed with sequence specific probes for eotaxin-1, eotaxin-2, and β -actin. Blots were placed in a phosphor imager autoradiography cassette and the phosphor screen was scanned with a Storm 860 scanner (Amersham Biosciences). Densitometry of the Northern blot was performed in Imagequant software and reported as a ratio of eotaxin expression to β -actin expression, which was similar in saline- and OVA-challenged mice. Data are expressed as the mean number of pixels above background divided by the mean number of pixels above background for β -actin (e.g., eotaxin/ β -actin) $\pm$ SEM.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical significance comparing different sets of mice was determined by Wilcoxon rank-sum of the mean test.

Results

Generation of eotaxin-2-deficient, eotaxin-1/2 DKO, and CCR3-deficient mice

To disrupt the eotaxin-2 gene (located on chromosome 5), a targeting strategy was used that deleted 1.86 kb of DNA corresponding to all of exons 1 and 2 (Fig. 1A) and thus producing a null mutation. Clones that underwent homologous recombination were screened by Southern blot analysis using the 3' probe. Two targeted clones were injected into blastocysts, and the resulting chimeras both transmitted the disrupted eotaxin-2 allele to progeny. Subsequently, eotaxin-2-deficient mice were crossed with eotaxin-1-deficient mice (located on chromosome 11) of the same 129 SvEv genetic background and F2 offspring were identified that contained deletions of both eotaxin genes (designated eotaxin-1/2 DKO).

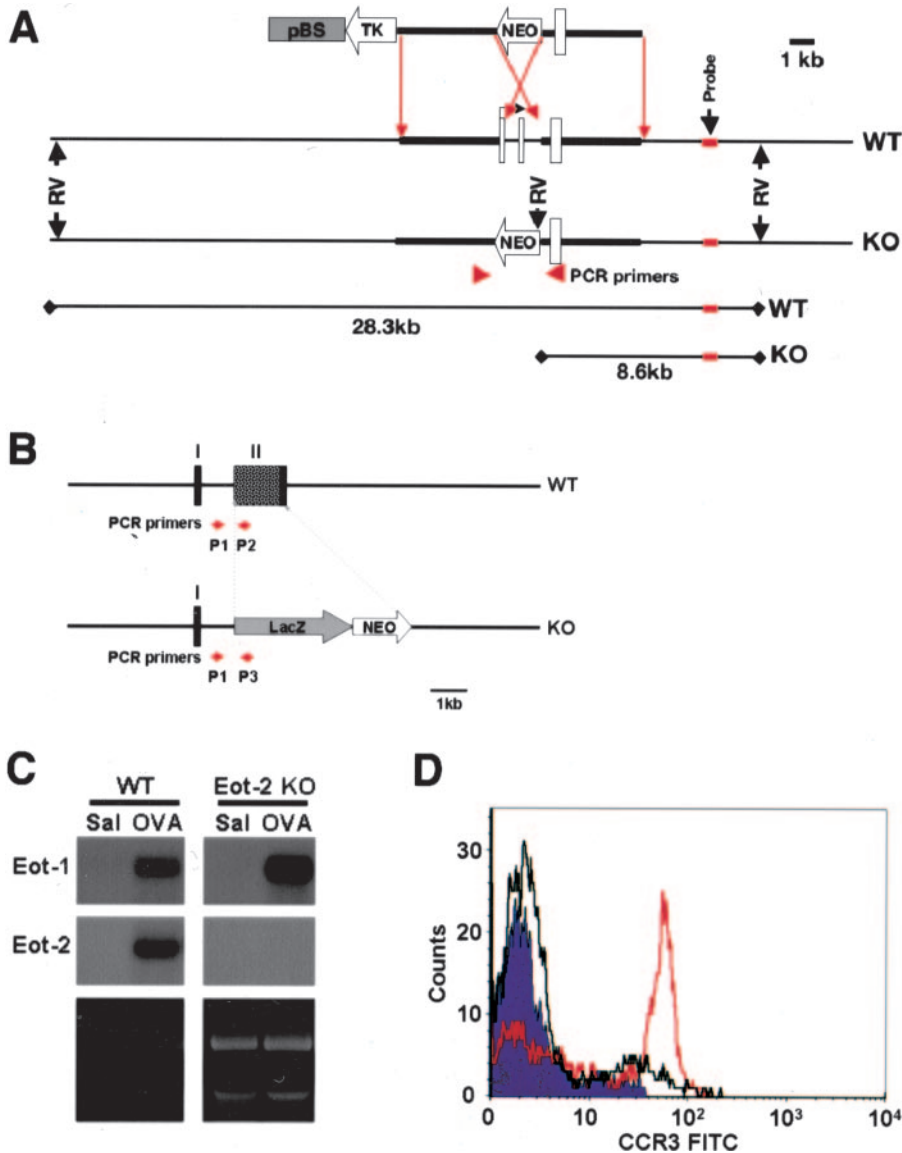
The murine CCR3 gene is composed of at least two exons, but importantly, the entire open reading frame of the gene product is present on a single exon (exon 2). To disrupt the *CCR3* gene, a targeting strategy was used that deleted 1074 bp of exon 2 (Fig. 1B). Embryonic stem cells containing CCR3-targeted clones were injected into blastocysts, and the disrupted CCR3 allele was found to be present in the germline of mice.

Cumulative genotyping of heterozygous crosses of mice containing either the eotaxin-2 or CCR3-targeted alleles revealed that the targeted alleles were inherited at predicted Mendelian ratios (data not shown). The eotaxin-2-targeting strategy was verified by the absence of eotaxin-2 in the BALF by ELISA (data not shown) and the absence of eotaxin-2 mRNA expression in the lungs of OVA-challenged mice (Fig. 1C). Notably, levels of eotaxin-1 were unaffected by disruption of the eotaxin-2 locus (Fig. 1C). Additionally, the CCR3-targeting strategy resulting in the loss of CCR3 expression was verified by Northern blot analysis of lung mRNA from OVA-challenged mice (data not shown) and by flow cytometric analysis (Fig. 1D).

Analysis of eotaxin-2-deficient, eotaxin-1/2 DKO, and CCR3-deficient mice revealed no gross or histological abnormalities in any organ, including those with abundant eotaxin-1 and/or eotaxin-2 expression (thymus, spleen, heart, and intestine). The leukocytes from the thymus and spleen from wild-type, eotaxin-2-deficient, eotaxin-1/2 DKO, and CCR3-deficient mice were subjected to flow cytometry analysis for cell surface markers. No quantitative or qualitative abnormalities were seen in leukocyte phenotype using lymphocyte markers that included B220, CD3, CD4, and CD8 (data not shown).

We first examined baseline eosinophil levels of the various gene-deficient mice in various hemopoietic tissues, including the blood, bone marrow, spleen, and jejunum (Table I). It is important to note for this and subsequent experiments that the CCR3-deficient mice were compared with their own matched wild-type control mice because the genetic background of this strain is not identical to the other mouse lines. This analysis revealed that eotaxin-2-deficient mice had normal levels of eosinophils in each compartment. In contrast, eotaxin-1/2 DKO mice had a 1.5- to 2-fold increase of eosinophils in the bone marrow and peripheral blood compared with wild-type mice ($p < 0.05$, $p < 0.01$) and a marked 85-fold fewer eosinophils in the jejunum ($p < 0.01$) (Table I). Although CCR3-deficient mice had similar numbers of eosinophils in the bone marrow, they had 2-fold fewer eosinophils in the peripheral blood compared with wild-type controls ($p < 0.05$) and over 150-fold fewer eosinophils in the jejunum ($p < 0.01$) (Table I). As a control, eotaxin-1-deficient mice had markedly reduced eosinophils in the jejunum (1.4 ± 0.4 eosinophils/mm²) ($p < 0.01$) compared with wild-type control mice. To investigate

FIGURE 1. Generation of eotaxin-2 and CCR3 gene-targeted mice. **A**, The generation of the eotaxin-2 gene-targeted mouse is depicted. Shown are the locations of the homologous arms used to generate the final construct, which were designed to replace NEO for exon 1 and exon 2 of eotaxin-2. Also shown are the locations of restriction enzyme site *EcoRV* (RV) and PCR primer sites used for genotyping. **B**, The VelociGene deletion strategy was used to delete the open reading frame of CCR3. Depicted are two exons of CCR3 and the protein coding region (start codon to stop codon) of exon 2 that is deleted. The positions of PCR primers for genotyping are also shown. **C**, Northern blot analysis of RNA from OVA-challenged wild-type and eotaxin-2-deficient mice using eotaxin-1 (Eot-1)- and eotaxin-2 (Eot-2)-specific probes show that eotaxin-2 induction is specifically ablated in eotaxin-2-deficient mice. The 18S and 28S bands of the ethidium bromide-stained gel are shown as a loading control. **D**, Flow cytometry of CCR3 expression specifically on gated spleen eosinophils of IL-5-induced, CCR3-deficient mice (black histogram) compared with wild-type mice (red histogram), and the filled histogram represents an isotype-matched control Ab.



the differences in eosinophil numbers in the bone marrow and blood of naive mice, we examined the numbers of eosinophil progenitor cells in the bone marrow. Interestingly, eosinophil progenitor numbers in eotaxin-1/2 DKO and CCR3 KO mice were comparable to levels seen in wild-type controls having 6.1 ± 1.4 and 4.5 ± 0.5 eosinophil CFU/ml, respectively (mean $\pm$ SEM; $n = 5$ and 4), compared with 6.6 ± 1.4 and 4.2 ± 0.4 eosinophil CFU/ml (mean $\pm$ SEM; $n = 5$ and 3) in their respective wild-type controls. Taken together, while there are subtle differences of eosinophil levels in the hemopoietic organs of mice with deletions of both eotaxin-1 and eotaxin-2 together and CCR3, the predominant ef-

fect of these genetic deletions is a marked deficiency of gastrointestinal eosinophils, a primary ramification of eotaxin-1.

Eotaxin 1/2 DKO and CCR3-deficient mice have elevated allergen-induced peripheral blood eosinophilia

To examine the role of the eotaxins and CCR3 in regulating eosinophil trafficking during the induction of experimental asthma, we subjected the various gene-targeted mice (and their strain-matched controls) to the OVA-induced pulmonary inflammation model. Analysis of peripheral blood from both strains of wild-type

Table I. Eosinophil levels in hematopoietic tissues and jejunum^a

Tissue	WT ^b	Eot-2 KO	Eot-1/2 DKO	CCR3 WT	CCR3 KO
Bone marrow (eos/ml $\times 10^5$)	5.2 $\pm$ 0.6	7.8 $\pm$ 1.1	9.9 $\pm$ 1.4 [†]	6.5 $\pm$ 1.6	9.6 $\pm$ 1.0
Blood (eos/ml $\times 10^5$)	1.5 $\pm$ 0.2	1.5 $\pm$ 0.2	3.2 $\pm$ 0.4*	1.3 $\pm$ 0.2	0.7 $\pm$ 0.1 [†]
Spleen (eos $\times 10^5$)	8.2 $\pm$ 1.2	7.7 $\pm$ 1.5	16.1 $\pm$ 6.1	12.9 $\pm$ 3.3	6.0 $\pm$ 1.1*
Jejunum (eos/mm ²)	366 $\pm$ 49	358 $\pm$ 64	4.3 $\pm$ 2.3*	146 $\pm$ 36	1.2 $\pm$ 0.8*

^a Results are depicted as the mean $\pm$ SEM ($n = 7-10$ for bone marrow; $n = 11-18$ for peripheral blood; $n = 7-14$ for spleen, and $n = 6-12$ for jejunum). An asterisk "*" indicates $p < 0.01$ and a "[†]" indicates $p < 0.05$ compared with wild-type mice.
^b WT, wild type.

mice revealed that eosinophil levels following two OVA challenges modestly decreased compared with saline-challenged mice. Interestingly, analysis of all of the gene-deficient mouse lines, except eotaxin-1-deficient mice, revealed consistent increases in OVA-induced blood eosinophilia compared with their wild-type controls. For example, eotaxin-2-deficient, eotaxin-1/2 DKO, and CCR3-deficient mice showed a 4-, 10-, and 6-fold increase over their wild-type controls, respectively, while peripheral blood eosinophil levels in OVA-challenged eotaxin-1-deficient mice were similar to wild-type mice (Fig. 2). There was a modest (not statistically significant) increase in the level of eosinophils in the saline-treated eotaxin-2 KO and eotaxin-1/2 DKO mice. Collectively, these results demonstrate that the eotaxin/CCR3 pathway can have effects on the level of allergen-induced eosinophil responses in the peripheral blood, likely by an indirect mechanism.

Eotaxin-1 and eotaxin-2 synergistically regulate allergen-induced airway eosinophilia

Examination of the OVA-challenged eotaxin-2-deficient mice revealed a ~4-fold reduction in the number of BALF eosinophils compared with OVA-challenged wild-type mice ($3.3 \pm 0.8 \times 10^5$ and $12 \pm 3.4 \times 10^5$, respectively) (mean $\pm$ SEM; $n = 15$ mice ($p < 0.02$)) (Fig. 3), which corresponded to a change in the percentage of eosinophils from 38.5 ± 14.0 and $57.8 \pm 16.5\%$ of the total cells in the BALF of eotaxin-2-deficient and wild-type mice, respectively (mean $\pm$ SEM; $n = 15$ mice ($p < 0.01$)). In contrast, eotaxin-1-deficient mice had levels of airway eosinophils similar to wild-type mice ($8.9 \pm 2.7 \times 10^5$) (Fig. 3). These results establish a dominant role for eotaxin-2 compared with eotaxin-1 in regulating OVA-induced BALF eosinophilia. Analysis of eotaxin-1/2 DKO mice showed a 7-fold decrease in BALF eosinophils compared with wild-type mice ($12 \pm 3.4 \times 10^5$ to $1.8 \pm 0.3 \times 10^5$, respectively ($p < 0.01$)). Notably, eotaxin-1/2 DKO mice had a decrease in BALF eosinophilia compared with mice carrying a deletion of eotaxin-1 or eotaxin-2 alone ($1.8 \pm 0.3 \times 10^5$ compared with $8.9 \pm 2.7 \times 10^5$ and $3.3 \pm 0.8 \times 10^5$, respectively ($p < 0.05$ and $p < 0.05$)) (Fig. 3). Analysis of CCR3-deficient mice revealed a consistent and profound decrease in OVA-induced

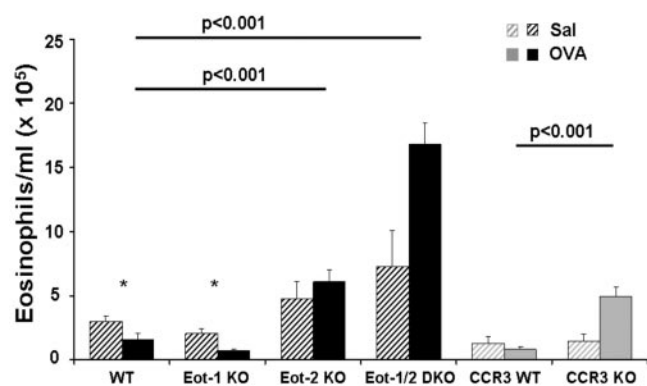


FIGURE 2. Peripheral blood eosinophil counts in saline and OVA-challenged mice. Wild-type (WT), eotaxin-1-deficient (Eot-1 KO), eotaxin-2-deficient (Eot-2 KO), eotaxin-1/2 DKO (Eot-1/2 DKO), CCR3 wild-type (CCR3 WT), and CCR3-deficient (CCR3 KO) mice were subjected to the OVA model of allergic airway inflammation. Blood was harvested after mice were sacrificed 18–20 h after the second challenge. Results show the mean eosinophils/milliliter per mouse $\pm$ SEM. An asterisk indicates values that have a Wilcoxon rank-sum value < 0.05 comparing OVA to the saline-challenged mice of the same genotype. (WT, $n = 9$ and 18; Eot-1 KO, $n = 4$ and 9; Eot-2 KO, $n = 6$ and 15; Eot-1/2 DKO, $n = 8$ and 20; CCR3 WT, $n = 13$ and 9; CCR3 KO, $n = 6$ and 18 for saline and OVA-challenged mice, respectively).

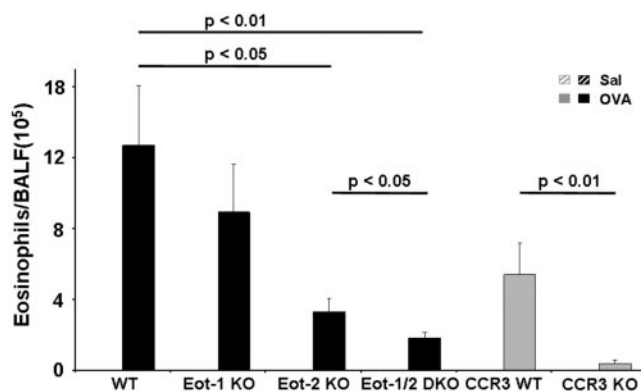


FIGURE 3. Eosinophil numbers in the BALF of OVA-challenged mice. Wild-type (WT), eotaxin-1-deficient (Eot-1 KO), eotaxin-2-deficient (Eot-2 KO), eotaxin-1/2 DKO (Eot-1/2 DKO), CCR3 wild-type (CCR3 WT), and CCR3-deficient (CCR3 KO) mice were subjected to the OVA model of allergic airway inflammation. Mice were sacrificed 18–20 h after the second challenge, and BALF was collected, and eosinophils were counted. All data are expressed as a mean number of eosinophils/BALF $\pm$ SEM (WT, $n = 9$ and 18; Eot-1 KO, $n = 4$ and 9; Eot-2 KO, $n = 6$ and 15; Eot-1/2 DKO, $n = 8$ and 20; CCR3 WT, $n = 6$ and 13; CCR3 KO, $n = 6$ and 18 for saline and OVA-challenged mice, respectively).

BALF eosinophilia. Analysis of noneosinophils in the BALF of allergen-challenged mice revealed no significant differences between the genetically engineered and wild-type mice (Table II).

Eotaxin-1 and eotaxin-2 synergistically regulate allergen-induced lung tissue eosinophilia

OVA-challenged eotaxin-1-deficient mice and eotaxin-2-deficient mice had levels of peribronchial eosinophils similar to wild-type mice (901 ± 93 , 622 ± 134 , and 717 ± 119 eosinophils/mm², respectively, mean $\pm$ SEM) (Fig. 4). However, there was a dramatic (85%) reduction of peribronchial eosinophils in the eotaxin-1/2 DKO mice compared with either eotaxin-1 or eotaxin-2 single deficiency alone ($p < 0.001$) being decreased from 901 ± 93 and 622 ± 134 eosinophils/mm² in eotaxin-1-deficient and eotaxin-2-deficient mice, respectively, to 106 ± 24 eosinophils/mm² in eotaxin-1/2 DKO (Fig. 4). This data suggest that the eotaxins act cooperatively to attract eosinophils and that ablation of both chemokines is required to significantly affect the level of tissue eosinophils. Furthermore, CCR3-deficient mice had an impressive reduction in the number of peribronchial eosinophils compared with wild-type mice (15.7 ± 5.1 vs 633 ± 149 eosinophils/mm², respectively) (Fig. 4). Representative photomicrographs from OVA-challenged wild-type, eotaxin-1/2 DKO, and CCR3-deficient mice are shown in Fig. 5. It is interesting to note that while wild-type mice and mice deficient in only one eotaxin chemokine contained eosinophils primarily in the peribronchial and perivascular regions, both the CCR3-deficient and eotaxin-1/2 DKO mouse lines showed only scattered eosinophils, primarily in the lung parenchyma not associated with the peribronchial/perivascular inflammatory infiltrates (Fig. 5 and data not shown).

Different induction patterns for eotaxin-1 and eotaxin-2

Collectively, our results substantiate cooperative and nonoverlapping roles for eotaxin-1 and eotaxin-2 in regulating OVA-induced inflammatory responses in the lung. We hypothesized that the eotaxins may have a distinct temporal and/or spatial induction profile. To test this hypothesis, we analyzed the kinetics of eotaxin-1 and eotaxin-2 accumulation in the lung between 0 and 120 h after the second allergen challenge. Northern blot analysis of eotaxin-1

Table II. Macrophage, neutrophil, and lymphocyte levels in the BALF^a

Genotype	Macrophages ($\times 10^4$)		Neutrophils ($\times 10^4$)		Lymphocytes ($\times 10^4$)	
	Sal	OVA	Sal	OVA	Sal	OVA
WT ^b	2.0 $\pm$ 0.3	34 $\pm$ 10	0.2 $\pm$ 0.1	17 $\pm$ 3.1	0.3 $\pm$ 0.1	15 $\pm$ 3.1
Eotaxin-1 KO	2.1 $\pm$ 0.3	33 $\pm$ 10	0.1 $\pm$ 0.02	11 $\pm$ 2.5	0.1 $\pm$ 0.02	10 $\pm$ 2.5
Eotaxin-2 KO	1.3 $\pm$ 0.4	22 $\pm$ 5.9	0.2 $\pm$ 0.1	12 $\pm$ 2.6	0.1 $\pm$ 0.1	9.0 $\pm$ 2.6
Eotaxin-1/2 DKO	4.4 $\pm$ 1.0	18 $\pm$ 3.1	0.3 $\pm$ 0.1	12 $\pm$ 2.6	1.5 $\pm$ 1.0	10 $\pm$ 2.6
CCR3 WT	12 $\pm$ 5.7	18 $\pm$ 4.0	1.2 $\pm$ 0.5	23 $\pm$ 5.6	0.3 $\pm$ 0.6	18 $\pm$ 5.6
CCR3 KO	7.1 $\pm$ 4.1	11 $\pm$ 3.0	0.4 $\pm$ 0.2	17 $\pm$ 5.3	0.5 $\pm$ 0.2	12 $\pm$ 5.2

^a The level of macrophages, neutrophils, and lymphocytes in the BALF 18–20 h after the second allergen challenge is shown. Results are depicted as the mean $\pm$ SEM ($n = 4$ –9 for saline and $n = 9$ –20 for OVA-challenged mice).

^b WT, wild type.

mRNA expression revealed peak levels at 6 h and a decline thereafter (Fig. 6A). Eotaxin-2 mRNA expression peaked somewhat later (10 h) and returned to baseline by 24 h (Fig. 6B). We next examined the level of eotaxin-1 and eotaxin-2 protein in the BALF. Eotaxin-1 protein peaked at 10 h and reached 0.15 ng/ml before declining to below the detection limit by 48 h (Fig. 6C). Statistically significant values ($p < 0.01$) for OVA compared with saline were observed at 6 and 10 h. Eotaxin-2 protein peaked at 10 h reaching 1.9 ng/ml (10-fold higher than eotaxin-1 levels) before declining to 0.6 ng/ml by 120 h (Fig. 6D). Statistically significant values ($p < 0.01$) were observed at 6, 10, and 24 h. Comparison of eotaxin induction with eosinophil accumulation in the BALF revealed that expression of the eotaxins preceded eosinophil accumulation, which peaks at 72 h and remains sustained for the duration of the analysis (Fig. 6E). There was not a statistically significant linear relationship between eotaxin-1 and -2 levels and eosinophil numbers in the BALF by Pearson's analysis. Thus, the largest difference between eotaxin-1 and eotaxin-2 protein expression in the BALF was the 10-fold higher eotaxin-2 concentration and the kinetics of protein decline.

Eotaxin-1 and eotaxin-2 are expressed in a distinct spatial compartment in the lung

To gain more mechanistic insight into the distinct roles of eotaxin-1 and eotaxin-2 in allergen-induced eosinophilia, we de-

termined the expression patterns of eotaxin-1 and eotaxin-2 in different compartments of the lung. As shown in Fig. 7, analysis of whole lung RNA revealed abundant levels of eotaxin-1 and eotaxin-2 mRNA in the OVA-challenged lung samples compared with saline control mice. However, in marked contrast to analysis of the whole lung, BALF cellular RNA selectively expressed eotaxin-2 mRNA. Thus, eotaxin-1 and eotaxin-2 are expressed in largely different locations in the allergic lung.

Eotaxin-2 is selectively expressed by lung macrophages/mononuclear cells

To examine the striking difference in the location of eotaxin-1 and eotaxin-2 expression, we performed in situ hybridization for eotaxin-2 mRNA expression. Hybridization of the eotaxin-2 antisense riboprobe to the OVA-challenged lung samples revealed

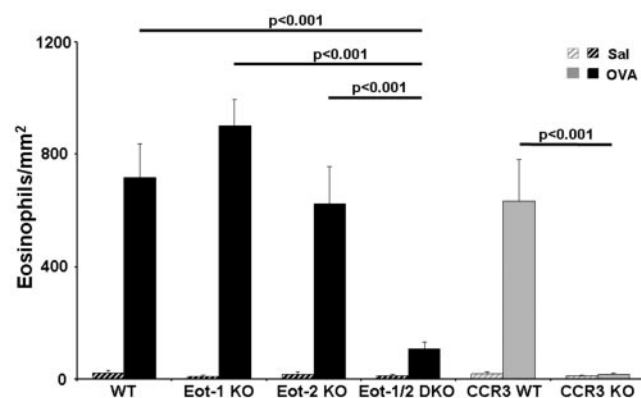


FIGURE 4. Eosinophil numbers in the peribronchial tissue of saline and OVA-challenged mice. Wild-type (WT), eotaxin-1-deficient (Eot-1 KO), eotaxin-2-deficient (Eot-2 KO), eotaxin-1/2 DKO (Eot-1/2 DKO), CCR3 wild-type (CCR3 WT), and CCR3-deficient (CCR3 KO) mice were subjected to the OVA model of allergic airway inflammation. Mice were sacrificed 18–20 h after the second challenge, and lung tissue was stained by anti-MBP immunohistochemistry and analyzed by morphometric analysis. All data are expressed as a mean number of eosinophils/mm² $\pm$ SEM. (WT, $n = 8$ and 20; Eot-1 KO, $n = 4$ and 9; Eot-2 KO, $n = 4$ and 14; Eot-1/2 DKO, $n = 6$ and 20; CCR3 WT, $n = 6$ and 11; CCR3 KO $n = 6$ and 16 for saline and OVA-challenged mice, respectively).

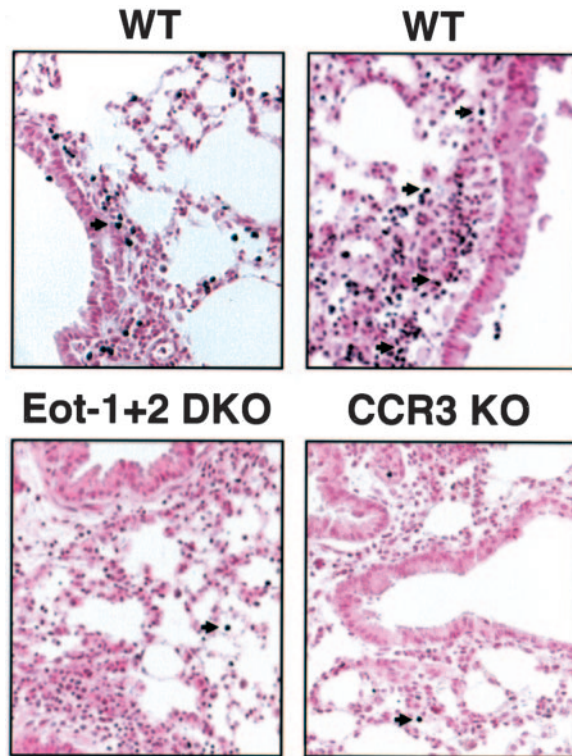


FIGURE 5. Photomicrographs of anti-MBP-stained lung sections. Lung tissue was collected from OVA-challenged wild-type (WT), eotaxin-1/2 DKO (Eot-1/2 DKO), and CCR3-deficient (CCR3 KO) mice 18–20 h after the second OVA challenge. Lung sections (5 μ m) were stained by anti-MBP immunohistochemistry identifying eosinophils as black stained cells. Photomicrographs were taken of representative airways with an original magnification of $\times 100$. Arrow points indicate representative eosinophils.

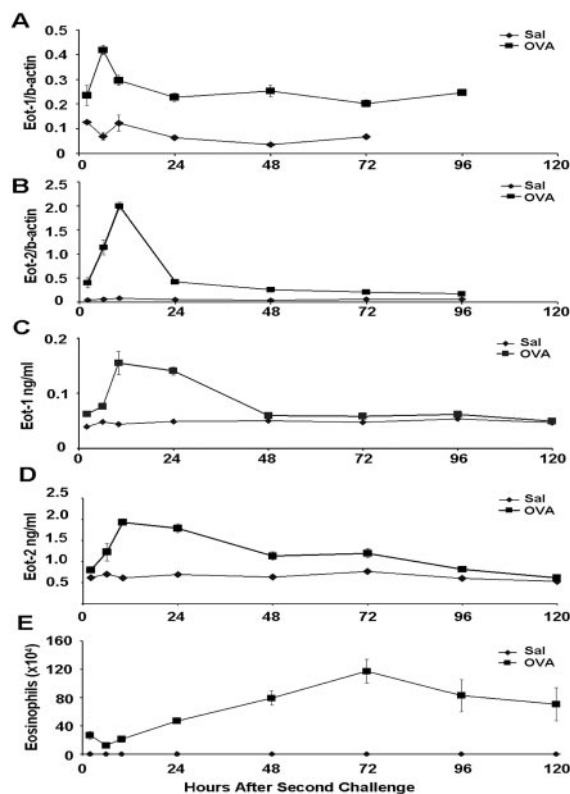


FIGURE 6. Eotaxin-1 and eotaxin-2 kinetic analysis. Wild-type mice were subjected to a model of OVA-induced pulmonary inflammation. Mice were sacrificed at the indicated time point after the second allergen challenge. *A* and *B*, The quantitative level of eotaxin-1 and eotaxin-2 mRNA expression in the lung is shown, respectively. For each time point, $n = 4$ for saline-challenged (Sal) mice and $n = 8$ for OVA-challenged (OVA) mice. *C*, The quantitative level of eotaxin-1 protein is shown. Data are expressed as the mean of the nanograms (ng) of eotaxin-1/milliliter of cell-free BALF supernatant $\pm$ SEM. Points < 150 pg/ml are extrapolated. For each time point, $n = 4$ for saline mice and $n = 8$ for OVA mice. *D*, The quantitative level of eotaxin-2 protein is shown. Points < 300 pg/ml are extrapolated. Data are expressed as the mean of the nanograms (ng) of eotaxin-2/milliliter of cell-free BALF supernatant $\pm$ SEM. For each time point, $n = 4$ for saline mice and $n = 8$ for OVA mice. *E*, The number of eosinophils counted in the BALF is shown. Data are expressed as the mean total eosinophils/mouse $\pm$ SEM. For each time point, $n = 4$ for saline mice and $n = 8$ for OVA mice.

strong staining in the peribronchial and perivascular regions, especially prominent in the inflammatory pockets (Fig. 8, *A* and *B* and data not shown). High-power magnification of the eotaxin-2 mRNA⁺ cells revealed that staining was present in mononuclear cells consistent with macrophages in the tissue and airway lumen, as shown by the representative light-field photomicrograph (Fig. 8, *C* and *D*). Interestingly, in situ hybridization for eotaxin-1 mRNA expression revealed similar strong staining in the peribronchial and perivascular regions (Fig. 8, *E* and *F*). However, in contrast to eotaxin-2 mRNA-positive cells and supporting Northern blot data, no eotaxin-1-positive cells were observed in the airway lumen. Examination under high-power magnification showed expression in dispersed mononuclear cells deep within the inflammatory infiltrate (Fig. 8, *E* and *F*). Indeed, upon quantitation there were 0.0 ± 0.0 eotaxin-1-positive cells/airway observed in medium sized airways compared with 0.23 ± 0.07 eotaxin-2-positive cells/airway (mean $\pm$ SEM; $n = 5$; $p < 0.05$). Control hybridization of the antisense probe to the saline-challenged lung samples or the sense-probe to the OVA-challenged lung samples revealed no dis-

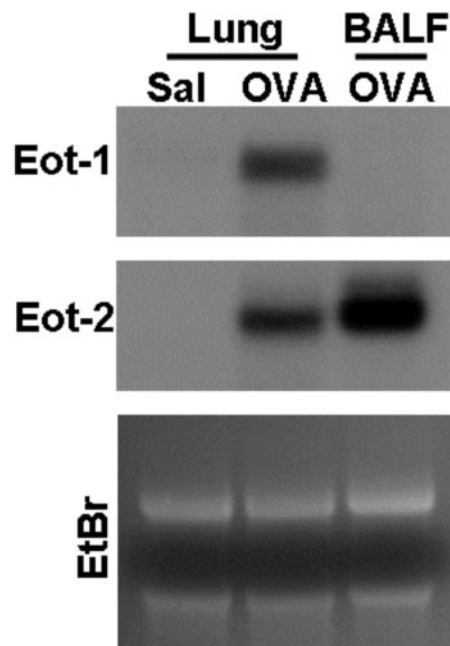


FIGURE 7. Eotaxin-1 and eotaxin-2 expression in BALF cells. BALF cells from OVA-challenged wild type mice were pooled from representative saline (Sal) or OVA-challenged wild-type mice. RNA (15 μ g) was then electrophoresed through 1.5% agarose, transferred to a hybridization membrane, and probed with sequence specific probes for eotaxin-1 and eotaxin-2. The 18S and 28S bands of the ethidium bromide (EtBr)-stained gel are shown as a loading control.

cernible signal for either eotaxin-1 or eotaxin-2 probes (data not shown).

Discussion

Eosinophilia has long been recognized as an important pathogenic factor associated with allergic airway inflammation (43). Thus, the mechanism of eosinophil recruitment to allergic tissue has been an area of intense research (44, 45). It has been appreciated that eosinophil recruitment to the lung is chemokine dependent (9). Indeed, the involvement of eotaxin-1 and eotaxin-2 has been described using Abs to these chemokines (46). However, the relative contribution of specific chemokines and nonchemokine chemoattractants (e.g., leukotrienes) has not been established. In the present study, we have used a standard mouse model of OVA-induced pulmonary inflammation to elucidate the role of the eotaxin/CCR3 pathway in eosinophil recruitment to the lung. To accomplish this goal, we generated novel eotaxin-2-deficient, eotaxin-1/2 DKO mice, and CCR3-deficient mice. Our results establish several fundamental principles concerning allergen-induced eosinophil trafficking to the lungs. First, our results establish that allergen-induced pulmonary eosinophilia is primarily mediated by CCR3 and its ligands eotaxin-1 and eotaxin-2 as evidenced by the profound synergistic reductions of airway and tissue eosinophilia in OVA-challenged eotaxin-1/2 DKO and CCR3-deficient mice compared with wild-type controls. This is a surprising observation because there are a variety of other chemoattractants implicated in eosinophil homing to the allergic lung including lipid mediators (e.g., leukotrienes (47), acidic mammalian chitinase (10), and 5-oxo-ETE (48)) and cytokines (e.g., IL-5 (17) and IL-16 (49)). Perhaps the observed dominant effect of the eotaxins and CCR3 in our model can be explained as being prerequisite for some of these other molecules to exert their effects. There are two

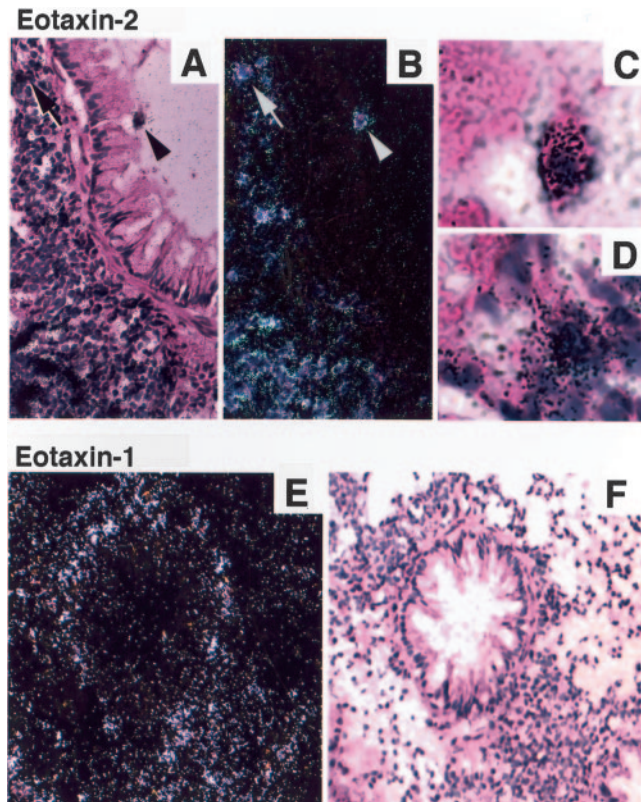


FIGURE 8. In situ hybridization for eotaxin-1 and eotaxin-2 in lung tissue. In situ hybridization for eotaxin-1 and eotaxin-2 mRNA was performed using the photographic overlay method, where positive signal appears in dark field microscopy as bright pink or white grains on a black background and in bright field microscopy as black precipitated granules in the overlayment. **A**, A representative bright field micrograph taken at $\times 100$ original magnification shows OVA-challenged lung airway probed with the antisense strand for eotaxin-2. **B**, The same airway is shown in a dark field micrograph at $\times 100$ original magnification. Please note the arrowhead and arrow indicating eotaxin-2-positive cells in the airway and peribronchial area, respectively. **C**, The cell indicated by the arrowhead is shown as a $\times 400$ bright field micrograph, and **D** shows the cell indicated by the arrow as a $\times 400$ bright field micrograph. **E**, A representative airway from an eotaxin-1 antisense strand probed OVA-challenged wild-type mouse lung is shown in a dark field micrograph ($\times 100$ original magnification), showing strong signal (white) surrounding the airway. **F**, A bright field micrograph shows the same area as **E**. Positive signal (black granules).

reports in the literature using an OVA model of pulmonary inflammation in CCR3-deficient mice. Notably, the CCR3-deficient mice in these studies were of a different genetic background and gene targeting strategy. One report, using a slightly different systemic sensitization protocol than our present study, indicated a nominal role for CCR3 in the development of airway eosinophilia after one challenge with aerosolized OVA (31). The other report used an epicutaneous model of allergen sensitization and found substantial decreases in airway and tissue eosinophils in CCR3-deficient mice after one aerosolized OVA challenge (30). Although the authors suggest that the distinct sensitization protocols were responsible for the observed differences, our results show that CCR3-dependent airway inflammation is not restricted to an epicutaneous sensitization. Importantly, while there are some residual eosinophils in the BALF and lung tissue observed in eotaxin-1/2 DKO and CCR3-deficient mice, the profound reduction of eosinophilia overshadows contributions by other factors reported to be important in eosinophil recruitment, including other chemokines (e.g., RANTES) (50). A second fundamental principle established

by our results is that eotaxin-2 has a dominant role in the development of OVA-induced airway eosinophilia as evidenced by the notably greater reduction of airway eosinophils in mice deficient in eotaxin-2 vs eotaxin-1 compared with wild-type mice. Notably, eotaxin-1-deficient mice have been shown previously to have a modest decrease in BALF eosinophils only after one intranasal OVA challenge (27). We also present mechanistic data concerning the differential role of eotaxin-1 and -2 in eosinophil recruitment to the lung. Although both eotaxin-1 and eotaxin-2 are expressed in the lung tissue, the dominant role of eotaxin-2 in regulating airway eosinophilia appears to be a ramification of the selective expression of eotaxin-2 by cells in the BALF, including macrophages/mononuclear cells. These results are in agreement with the role and expression pattern of eotaxin-2 in IL-13-induced pulmonary eosinophilia (33). Furthermore, the dominant role of eotaxin-2 in the airway is supported by the observation that the magnitude of the increase of eotaxin-2 protein in the BALF was greater than for eotaxin-1 protein and that eotaxin-1 protein already returned to control levels by 48 h. In addition, the peak concentration of eotaxin-2 in the BALF was >10 -fold higher than that of eotaxin-1. Although binding affinities for eotaxin-1 and -2 for CCR3 have not been published for the murine proteins, human eotaxin-1 and eotaxin-2 competitive binding studies show identical IC_{50} values (51). However, in the mouse, eotaxin-1 has a 5-fold higher chemotactic activity at 10 ng/ml; the minimal concentration to induce eosinophil chemotaxis is similar for eotaxin-1 and -2 (20). Combined with other studies identifying eotaxin-1 and -2 induction to be STAT6 dependent (9), these collective results strongly support the identification of macrophage/monocyte-derived eotaxin-2 as the critical STAT6-dependent eosinophil chemoattractant responsible for allergen-induced airway eosinophilia. Interestingly, activated STAT6 alone within a cell must not be sufficient to activate transcription of eotaxin-1 in the infiltrating cells that are found in the airways of OVA-challenged mice.

Our findings, showing differential eotaxin-1 and -2 expression and subsequent eosinophil sublocalization explain and expand reports in human subjects. Although eotaxin-3 may contribute to pulmonary eosinophilia in humans (52), there are still striking similarities between mouse and human. Our finding that infiltrating macrophages/mononuclear cells are a source of eotaxin-2 corroborate reports showing that human peripheral blood monocytes produce high levels of eotaxin-2 (53). Clinical studies have demonstrated kinetic induction patterns of eotaxin-1 and eotaxin-2 similar to those observed in our model, where eotaxin-1 mRNA expression peaked at 6 h, whereas eotaxin-2 peaked at 24 h following cutaneous allergen challenge (54). Our results extend the human data by showing differential eotaxin expression is functionally linked to observed eosinophil numbers and that both eotaxin chemokines cooperatively regulate the regional localization of allergen-induced lung tissue eosinophilia.

A third fundamental principle established by our data is a cooperative role for eotaxin-1 and eotaxin-2 in the recruitment of eosinophils to the lung tissue as demonstrated by substantial reductions in peribronchial eosinophilia and lack of spatial organization of the remaining lung tissue eosinophils in eotaxin-1/2 DKO and CCR3-deficient mice. Cooperativity of eotaxin-1 and -2 was also supported by in situ hybridization showing similar but also nonoverlapping staining patterns for both eotaxins that correlated with eosinophil localization in the lung. Notably, eotaxin-1 expression was limited to the tissue, supporting prior studies in an IL-13-induced model of allergic airway inflammation showing eotaxin-1 to be important in the development and/or maintenance

of peribronchial eosinophilia (17), whereas eotaxin-2 was primarily responsible for IL-13-induced airway eosinophilia (33). Additionally, in mice lacking one eotaxin, there was no compensatory overexpression of the remaining eotaxin chemokine (33). Finally, our results indicate a regulatory role for eotaxin-2 in the development of peripheral blood eosinophilia, indicated by increased peripheral blood eosinophilia in OVA-challenged eotaxin-2-deficient, eotaxin-1/2 DKO, and CCR3-deficient mice. Thus, mice that were able to produce and/or respond to eotaxin-2 (wild-type and eotaxin-1-deficient mice) did not develop OVA-induced peripheral blood eosinophilia. Although it is most likely that the role of eotaxin-2 in regulating blood eosinophilia is secondary to its dominant role in recruitment of eosinophils from the peripheral blood into the tissue, it is noteworthy that eotaxin-2 was first described as a myeloid progenitor inhibitory factor (18). Although it is intriguing to speculate that eotaxin-2 and CCR3 might have a role in eosinophilopoiesis ultimately affecting peripheral blood eosinophil levels, our CFU data in vitro does not support a primary role for these molecules in regulating eosinophil development, consistent with only modest changes in eosinophil levels seen in various hematopoietic tissues in the eotaxin-1/2 DKO and CCR3 KO mice.

In summary, our data establishes that 1) allergen-induced pulmonary eosinophilia is primarily mediated by CCR3 and its ligands eotaxin-1 and eotaxin-2; 2) eotaxin-2 has a dominant role in OVA-induced airway eosinophilia mediated by expression of eotaxin-2 by cells found in the BALF, including macrophages and mononuclear cells; 3) a cooperative role for eotaxin-1 and eotaxin-2 in recruitment of eosinophils to the lung tissue; and 4) a regulatory role for eotaxin-2 in the development of peripheral blood eosinophilia.

Based on these results, an agent that could block eotaxin-1 and eotaxin-2 interaction with CCR3 is likely to effectively reduce the number of eosinophils that migrate into the lung. A prior study has suggested that CCR3 ablation may promote mast cell responses (31); however, we did not observe any changes in tracheal mast cell numbers (mast cells/mm² were 49.0 ± 24.7 , 51.6 ± 24.5 , 34.7 ± 14.6 , and 57.0 ± 28.8 for OVA-challenged wild-type, Eot-1/2 KO, CCR3 wild-type, and CCR3 KO mice, respectively; $n = 4$). We also did not observe any differences in mucus production in the various genetically deficient mice compared with wild-type (data not shown).

We have not yet been able to examine the impact of these genetic deletions on the development of airway hyperresponsiveness because we were unable to elicit airway hyperresponsiveness (as measured by whole body plethysmography) in 129/SvEv mice using our present experimental protocol (S. M. Pope and M. E. Rothenberg, unpublished results). It will be interesting to examine the impact of eotaxin-1 and eotaxin-2 on airway hyperresponsiveness once we have completed backcrossing our mice onto a BALB/c genetic background that is known to develop airway hyperresponsiveness under our experimental conditions. Interestingly, our data also suggest a therapeutic agent that could effectively block eotaxin-2 from interacting with CCR3 during an allergic response might have the undesirable effect of generating peripheral blood eosinophilia. Thus, such therapy might be more beneficial if used in combination with heretofore disappointing anti-IL-5 therapy, which has been shown to reduce peripheral blood eosinophilia, but has had only limited ability to reduce tissue eosinophilia or allergic symptoms such as bronchoconstriction and airway hyper-reactivity (13). Interestingly, identification of macrophages in the airway as a source of eotaxin-2 elucidates a speculation made by Voehringer et al. (26), where it was reported that STAT6-competent bone marrow-derived (non-T or B) cells were required mediators of pulmonary eosinophilia. Taken together, it is

clear that eotaxin-1 and eotaxin-2 are the major ligands involved in CCR3-mediated eosinophil migration to the lung in this model of allergen-induced pulmonary eosinophilia, and as such, it would appear that targeting multiple CCR3 ligands would be a therapeutic strategy worthy of testing.

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Disclosures

The authors have no financial conflict of interest.

References

- Lee, J. J., D. Dimina, M. P. Macias, S. I. Ochkur, M. P. McGarry, K. R. O'Neill, C. Protheroe, R. Pero, T. Nguyen, S. A. Cormier, et al. 2004. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305: 1773–1776.
- Humbles, A. A., C. M. Lloyd, S. J. McMillan, D. S. Friend, G. Xanthou, E. E. McKenna, S. Ghiran, N. P. Gerard, C. Yu, S. H. Orkin, and C. Gerard. 2004. A critical role for eosinophils in allergic airways remodeling. *Science* 305: 1776–1779.
- Gleich, G. J. 2000. Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* 105: 651–663.
- Broide, D., and P. Sriramarao. 2001. Eosinophil trafficking to sites of allergic inflammation. *Immunol. Rev.* 179: 163–172.
- Moser, R., J. Fehr, and P. L. Bruijnzeel. 1992. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J. Immunol.* 149: 1432–1438.
- Sher, A., R. L. Coffman, S. Hieny, P. Scott, and A. W. Cheever. 1990. Interleukin 5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with *Schistosoma mansoni*. *Proc. Natl. Acad. Sci. USA* 87: 61–65.
- Horie, S., Y. Okubo, M. Hossain, E. Sato, H. Nomura, S. Koyama, J. Suzuki, M. Isobe, and M. Sekiguchi. 1997. Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. *Intern. Med.* 36: 179–185.
- Weber, C., J. Kitayama, and T. A. Springer. 1996. Differential regulation of β_1 and β_2 integrin avidity by chemoattractants in eosinophils. *Proc. Natl. Acad. Sci. USA* 93: 10939–10944.
- Zimmermann, N., G. K. Hershey, P. S. Foster, and M. E. Rothenberg. 2003. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J. Allergy Clin. Immunol.* 111: 227–42; quiz 243.
- Zhu, Z., T. Zheng, R. J. Homer, Y. K. Kim, N. Y. Chen, L. Cohn, Q. Hamid, and J. A. Elias. 2004. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 304: 1678–1682.
- Rankin, S. M., D. M. Conroy, and T. J. Williams. 2000. Eotaxin and eosinophil recruitment: implications for human disease. *Mol. Med. Today* 6: 20–27.
- Collins, P. D., S. Marleau, D. A. Griffiths-Johnson, P. J. Jose, and T. J. Williams. 1995. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J. Exp. Med.* 182: 1169–1174.
- Flood-Page, P. T., A. N. Menzies-Gow, A. B. Kay, and D. S. Robinson. 2003. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am. J. Respir. Crit. Care Med.* 167: 199–204.
- Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183: 195–201.
- Rothenberg, M. E., A. D. Luster, C. M. Lilly, J. M. Drazen, and P. Leder. 1995. Constitutive and allergen-induced expression of eotaxin mRNA in the guinea pig lung. *J. Exp. Med.* 181: 1211–1216.
- Yang, M., S. P. Hogan, S. Mahalingam, S. M. Pope, N. Zimmermann, P. Fulkerson, L. A. Dent, I. G. Young, K. I. Matthaei, M. E. Rothenberg, and P. S. Foster. 2003. Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperactivity. *J. Allergy Clin. Immunol.* 112: 935–943.
- Pope, S. M., E. B. Brandt, A. Mishra, S. P. Hogan, N. Zimmermann, K. I. Matthaei, P. S. Foster, and M. E. Rothenberg. 2001. IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J. Allergy Clin. Immunol.* 108: 594–601.
- Patel, V. P., B. L. Kreider, Y. Li, H. Li, K. Leung, T. Salcedo, B. Nardelli, V. Pippalla, S. Gentz, R. Thotakura, et al. 1997. Molecular and functional characterization of two novel human C-C chemokines as inhibitors of two distinct classes of myeloid progenitors. *J. Exp. Med.* 185: 1163–1172.

19. Shinkai, A., H. Yoshisue, M. Koike, E. Shoji, S. Nakagawa, A. Saito, T. Takeda, S. Imabeppu, Y. Kato, N. Hanai, et al. 1999. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. *J. Immunol.* 163: 1602–1610.
20. Zimmermann, N., S. P. Hogan, A. Mishra, E. B. Brandt, T. R. Bodette, S. M. Pope, F. D. Finkelman, and M. E. Rothenberg. 2000. Murine eotaxin-2: a constitutive eosinophil chemokine induced by allergen challenge and IL-4 overexpression. *J. Immunol.* 165: 5839–5846.
21. Lukacs, N. W., A. L. Miller, and C. M. Hogaboam. 2003. Chemokine receptors in asthma: searching for the correct immune targets. *J. Immunol.* 171: 11–15.
22. Daugherty, B. L., S. J. Siciliano, J. A. DeMartino, L. Malkowitz, A. Sirotina, and M. S. Springer. 1996. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J. Exp. Med.* 183: 2349–2354.
23. Gerber, B. O., M. P. Zanni, M. Uguccioni, M. Loetscher, C. R. Mackay, W. J. Pichler, N. Yawalkar, M. Baggiolini, and B. Moser. 1997. Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. *Curr. Biol.* 7: 836–843.
24. de Paulis, A., F. Annunziato, L. Di Gioia, S. Romagnani, M. Carfora, C. Beltrame, G. Marone, and P. Romagnani. 2001. Expression of the chemokine receptor CCR3 on human mast cells. *Int. Arch. Allergy Immunol.* 124: 146–150.
25. Mould, A. W., A. J. Ramsay, K. I. Matthaei, I. G. Young, M. E. Rothenberg, and P. S. Foster. 2000. The effect of IL-5 and eotaxin expression in the lung on eosinophil trafficking and degranulation and the induction of bronchial hyperactivity. *J. Immunol.* 164: 2142–2150.
26. Voehringer, D., K. Shinkai, and R. M. Locksley. 2004. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20: 267–277.
27. Rothenberg, M. E., J. A. MacLean, E. Pearlman, A. D. Luster, and P. Leder. 1997. Targeted disruption of the chemokine eotaxin partially reduces antigen induced tissue eosinophilia. *J. Exp. Med.* 185: 785–790.
28. Swiatek, P. J., and T. Gridley. 1993. Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. *Genes Dev.* 7: 2071–2084.
29. Gale, N. W., P. Baluk, L. Pan, M. Kwan, J. Holash, T. M. DeChiara, D. M. McDonald, and G. D. Yancopoulos. 2001. Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. *Dev. Biol.* 230: 151–160.
30. Ma, W., P. J. Bryce, A. A. Humbles, D. Laouini, A. Yalcindag, H. Alenius, D. S. Friend, H. C. Oettgen, C. Gerard, and R. S. Geha. 2002. CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J. Clin. Invest.* 109: 621–628.
31. Humbles, A. A., B. Lu, D. S. Friend, S. Okinaka, J. Lora, A. Al-Garawi, T. R. Martin, N. P. Gerard, and C. Gerard. 2002. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc. Natl. Acad. Sci. USA* 99: 1479–1484.
32. Luster, A. D. 2001. Antichemokine immunotherapy for allergic diseases. *Curr. Opin. Allergy Clin. Immunol.* 1: 561–567.
33. Pope, S. M., P. C. Fulkerson, C. Blanchard, H. Saito Akei, N. M. Nikolaidis, N. Zimmermann, J. D. Molkentin, and M. E. Rothenberg. 2005. Identification of a cooperative mechanism involving IL-13 and eotaxin-2 in experimental allergic lung inflammation. *J. Biol. Chem.* 280: 13952–13961.
34. Valenzuela, D. M., A. J. Murphy, D. Friendewey, N. W. Gale, A. N. Economides, W. Auerbach, W. T. Poueymirou, N. C. Adams, J. Rojas, J. Yasenchak, et al. 2003. High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. *Nat. Biotechnol.* 21: 652–659.
35. Fedorov, L. M., H. Haegel-Kronenberger, and J. Hirschhain. 1997. A comparison of the germline potential of differently aged ES cell lines and their trans-fected descendants. *Transgenic Res.* 6: 223–231.
36. Mishra, A., T. E. Weaver, D. C. Beck, and M. E. Rothenberg. 2001. Interleukin-5-mediated allergic airway inflammation inhibits the human surfactant protein C promoter in transgenic mice. *J. Biol. Chem.* 276: 8453–8459.
37. Brandt, E. B., and M. E. Rothenberg. 2001. Eosinophil levels in mice are significantly higher in small blood vessels than in large blood vessels. *J. Allergy Clin. Immunol.* 108: 142–143.
38. Saito, H., K. Howie, J. Wattie, A. Denburg, R. Ellis, M. D. Inman, and J. A. Denburg. 2001. Allergen-induced murine upper airway inflammation: local and systemic changes in murine experimental allergic rhinitis. *Immunology* 104: 226–234.
39. Mould, A. W., K. I. Matthaei, I. G. Young, and P. S. Foster. 1997. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J. Clin. Invest.* 99: 1064–1071.
40. Matthews, A. N., D. S. Friend, N. Zimmermann, M. N. Sarafi, A. D. Luster, E. Pearlman, S. E. Wert, and M. E. Rothenberg. 1998. Eotaxin is required for the baseline level of tissue eosinophils. *Proc. Natl. Acad. Sci. USA* 95: 6273–6278.
41. Friend, D. S., N. Ghildyal, K. F. Austen, M. F. Gurish, R. Matsumoto, and R. L. Stevens. 1996. Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J. Cell Biol.* 135: 279–290.
42. Zimmermann, N., N. E. King, J. Laporte, M. Yang, A. Mishra, S. M. Pope, E. E. Muntel, D. P. Witte, A. A. Pegg, P. S. Foster, Q. Hamid, and M. E. Rothenberg. 2003. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J. Clin. Invest.* 111: 1863–1874.
43. Wenzel, S. 2003. Mechanisms of severe asthma. *Clin. Exp. Allergy* 33: 1622–1628.
44. Adamko, D., P. Lacy, and R. Moqbel. 2004. Eosinophil function in allergic inflammation: from bone marrow to tissue response. *Curr. Allergy Asthma Rep.* 4: 149–158.
45. Gerard, C., and B. J. Rollins. 2001. Chemokines and disease. *Nat. Immunol.* 2: 108.
46. Radinger, M., A. K. Johansson, B. Sitkauskienė, M. Sjostrand, and J. Lotvall. 2004. Eotaxin-2 regulates newly produced and CD34 airway eosinophils after allergen exposure. *J. Allergy Clin. Immunol.* 113: 1109–1116.
47. Vargaftig, B. B., and M. Singer. 2003. Leukotrienes mediate murine broncho-pulmonary hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukin-13. *Am. J. Respir. Cell. Mol. Biol.* 28: 410–419.
48. Schwenk, U., and J. M. Schroder. 1995. 5-Oxo-eicosanoids are potent eosinophil chemotactic factors: functional characterization and structural requirements. *J. Biol. Chem.* 270: 15029–15036.
49. Okubo, Y., A. Tsukadaira, S. Takashi, K. Kubo, and S. Koyama. 2001. Chemotaxis of human CD4⁺ eosinophils. *Int. Arch. Allergy Immunol.* 125(Suppl. 1): 19–21.
50. Lukacs, N. W., R. M. Strieter, K. Warmington, P. Lincoln, S. W. Chensue, and S. L. Kunkel. 1997. Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J. Immunol.* 158: 4398–4404.
51. White, J. R., C. Imburgia, E. Dul, E. Appelbaum, K. O'Donnell, D. J. O'Shannessy, M. Brawner, J. Fornwald, J. Adamou, N. A. Elshourbagy, et al. 1997. Cloning and functional characterization of a novel human CC chemokine that binds to the CCR3 receptor and activates human eosinophils. *J. Leukoc. Biol.* 62: 667–675.
52. Berkman, N., S. Ohnana, F. K. Chung, and R. Breuer. 2001. Eotaxin-3 but not eotaxin gene expression is up-regulated in asthmatics 24 hours after allergen challenge. *Am. J. Respir. Cell Mol. Biol.* 24: 682–687.
53. Watanabe, K., P. J. Jose, and S. M. Rankin. 2002. Eotaxin-2 generation is differentially regulated by lipopolysaccharide and IL-4 in monocytes and macrophages. *J. Immunol.* 168: 1911–1918.
54. Ying, S., D. S. Robinson, Q. Meng, L. T. Barata, A. R. McEuen, M. G. Buckley, A. F. Walls, P. W. Askenase, and A. B. Kay. 1999. C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). *J. Immunol.* 163: 3976–3984.