Identification of a Cooperative Mechanism Involving Interleukin-13 and Eotaxin-2 in Experimental Allergic Lung Inflammation*

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Pulmonary eosinophilia, a hallmark pathologic feature of allergic lung disease, is regulated by interleukin-13 (IL-13) as well as the eotaxin chemokines, but the specific role of these cytokines and their cooperative interaction are only partially understood. First, we elucidated the essential role of IL-13 in the induction of the eotaxins by comparing IL-13 gene-targeted mice with wild type control mice by using an ovalbumin-induced model of allergic airway inflammation. Notably, ovalbumin-induced expressions of eotaxin-1 and eotaxin-2 mRNA in the lungs were almost completely dependent upon IL-13. Second, in order to address the specific role of eotaxin-2 in IL-13-induced pulmonary eosinophilia, we generated eotaxin-2 gene-deficient mice by homologous recombination. Notably, in contrast to observations made in eotaxin-1-deficient mice, eotaxin-2-deficient mice had normal base-line eosinophil levels in the hematopoietic tissues and gastrointestinal tract. However, following intratracheal IL-13 administration, eotaxin-2-deficient mice showed a profound reduction in airway eosinophilia compared with wild type mice. Most interestingly, the level of peribronchial lung tissue eosinophils in IL-13-treated eotaxin-2-deficient mice was indistinguishable from wild type mice. Furthermore, IL-13 lung transgenic mice genetically engineered to be deficient in eotaxin-2 had a marked reduction of luminal eosinophils. Mechanistic analysis identified IL-13-induced eotaxin-2 expression by macrophages in a distinct lung compartment (luminal inflammatory cells) compared with eotaxin-1, which was expressed solely in the tissue. Taken together, these results demonstrate a cooperative mechanism between IL-13 and eotaxin-2. In particular, IL-13 mediates allergen-induced eotaxin-2 expression, and eotaxin-2 mediates IL-13-induced airway eosinophilia.

There is currently an epidemic of allergic diseases in the western world (1). Experimentation in the asthma field has largely focused on analysis of the cellular and molecular events induced by allergen exposure in sensitized animals (primarily mice) and humans. These studies have identified elevated production of IgE, airway obstruction, eosinophil inflammation, enhanced bronchial reactivity to spasmogens, epithelial metaplasia (including goblet cell formation), and epithelial injury in the asthmatic response (2). Clinical and experimental investigations have demonstrated a strong correlation between the presence of CD4⁺ T helper 2 lymphocytes (Th2 cells), lung eosinophilia, and disease severity, suggesting an integral role for these cells in the pathophysiology of asthma (3, 4). Th2 cells are thought to induce asthma through the secretion of an array of cytokines (IL-4, -5, -9, and -13) that activate inflammatory and resident effector pathways both directly and indirectly (5). Eosinophils are thought to contribute to allergic airway inflammation by release of their pro-inflammatory cytotoxic granule proteins, generation of lipid mediators such as the cysteinyl-leukotrienes, and their release of mediators that can induce lung remodeling (e.g. transforming growth factors) (6, 7). Accordingly, elucidating the mechanism of Th2 cell and eosinophil recruitment and the cooperative role of these cells in the induction of allergic airway inflammation is an active area of research investigation and therapeutic intervention (8–10). The importance of elucidating the molecular mechanisms that govern eosinophil trafficking to the lung has been recently reinforced by the dramatic protection from experimental asthma seen in two different eosinophil-deficient mouse lines (11, 12).

Eosinophil recruitment into inflammatory sites is a complex process regulated by a number of cytokines, including IL-3, granulocyte macrophage colony-stimulating factor, chemokines (e.g. regulated on activation normal T cell expressed and secreted and the eotaxins), and Th2 cell products (e.g. IL-4, IL-5, IL-9, and IL-13) (13–15). IL-13 is particularly important because it is produced in high quantities by Th2-cells and regulates multiple features of asthma (IgE production, mucus overproduction, eosinophil recruitment and survival, airway hyper-responsiveness (AHR), and the expression of CD23, adhesion molecules, and chemokines (e.g. eotaxin)) (16, 17). Dysregulation of IL-13 has been reported in a variety of medical problems, including asthma, chronic obstructive pulmonary
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disease (18), and atopic dermatitis (19–25). Overexpression of IL-13, by pharmacological administration or transgenic approaches, induces multiple features of asthma, including eosphhilosis, mucus overproduction, and AHR, that appear to be mediated, at least in part, by chemokine induction (26, 27). During induction of IL-13-induced eosinophil-associated allergic airway inflammation, it has been proposed that the effect of IL-13 may be chemokine-dependent, as demonstrated by reduced lung inflammation in IL-13 lung transgenic mice engineered to be deficient in the chemokine receptor CCR2 (28). In addition to inducing CCR2 ligands, focusing on eosinophils, a paradigm has emerged implicating IL-4 and IL-13 in the induction of eosinophil active chemokines (29). In particular, IL-4 and IL-13 signaling involves utilization of the IL-4 receptor α-chain (30, 31) and the induction of Janus kinase 1 and signal transducers and activators of transcription-6 (32–35). However, the relative role of IL-4, IL-13, and other allergen-induced cytokines that have been shown to induce chemokines (e.g. tumor necrosis factor α) in the induction of the eotaxins has not been established. In addition, recent attention has been drawn to acidic mammalian chitinase in the regulation of eosinophilia and eotaxin induction (36). By using experimental models of antigen-induced airway inflammation, eotaxin-1 has been demonstrated to partially regulate eosinophil recruitment during the late phase response following repetitive mucosal antigen challenges (37, 38). Mice deficient in both eotaxin-1 and IL-5 have a synergistic deficiency of lung eosinophils compared with mice deficient in either cytokine alone (39). Also, eotaxin-1/IL-5 double-deficient mice have an intrinsic deficiency in IL-13 production by antigen-specific CD4+ cells, demonstrating that eotaxins, eotaxin-1, and IL-13 cooperate in allergic airway inflammation (40). Additionally, intratracheal delivery of eotaxin-2 to IL-5 transgenic mice induces enhanced pulmonary eosinophilia and AHR compared with delivery to wild type mice (41). Although much progress has been made in understanding the specific contribution of eotaxin-1 in allergic airway inflammation, the role of eotaxin-2 has been less extensively studied. Notably, eotaxin-2 was originally identified based on its functional effect as a selective chemotactic cell of eosinophils to the lung in a model of experimental asthma (42). However, both mouse and human eotaxin-2 were discovered by genomic approaches (43–45). Accordingly, there is less certainty concerning the role of eotaxin-2 in asthmatic responses. In this work we aimed to further characterize the interaction of IL-13 and eotaxin-2 in the induction of lung eosinophilia. First, we were interested in defining the role of IL-13 in allergen-induced expression of the eotaxins. Second, we were interested in testing the role of eotaxin-2 in the recruitment of eosinophils into the lung in response to IL-13. Our results establish a critical role for IL-13 in allergen-induced expression of the eotaxins. Additionally, we generated eotaxin-2 gene-targeted mice and used them to demonstrate an essential role for eotaxin-2 in mediating IL-13-induced airway eosinophilia. Furthermore, mechanistic analysis identified IL-13-induced eotaxin-2 expression in a distinct cellular compartment (airway lumen) compared with eotaxin-1, which was solely expressed by cells found in the lung tissue. Taken together, these results identify a linear relationship in which allergen induces IL-13, which in turn is required for eotaxin-2 induction, which in turn regulates airway eosinophilia in a manner distinct from eotaxin-1.

EXPERIMENTAL PROCEDURES

Mice—Mice were maintained under specific pathogen-free conditions and examined at 6–8 weeks of age. IL-13-deficient mice and matched control mice with a BALB/c background (F6) were kindly provided by Dr. Andrew McKenzie (46). Mice carrying the tetracycline-inducible IL-13 transgene under the regulation of the Clara cell 10 (CC10) lung promoter have been described previously (47). In brief, a two-transgenic system was used to target the expression of IL-13 to the lung in an externally regulated fashion. The activator mice, containing the CC10-tTA-hGH transgene, were kindly provided by Dr. Jeffrey Whitsett (Cincinnati Children’s Hospital Medical Center) (48). Mice carrying the TetO-CMVp-IL-13-hGH transgene were bred with activator mice, and the resulting bitransgenic mice (CC10-tTA-IL-13) at 4 weeks of age were fed doxycycline-impregnated food for 1–3 days. Generation of Eotaxin-2-deficient Mice—An eotaxin-2-targeting construct (Fig. IA) was constructed by cloning a 5’ genomic fragment containing a 4.0-kb-long PCR product (5’-ACTGAGTGCGTGATTGAGACCG-3’ and 3’-ACTGAGGAGGACGGACTTCAAC) by utilizing Expand™Long Template PCR system (Invitrogen) and a 3’ genomic fragment containing a 3.9-kb SmaI-EcoRI fragment into a pGKneo plasmid vector (Boehringer Mishina GmbH, Houston, TX), which was modified to have a herpes simplex virus-thymidine kinase gene inserted 3’ of the neomycin resistance gene. The genomic arms were derived from two 5’ phage DNA clones derived from 129 SvEv mice (Fig. 1A). The vector was linearized and electroporated into KG-1 embryonic stem cells of the 129 SvEv strain, and 288 neomycin-resistant clones were screened by Southern blot analysis by using a 622-bp PCR-generated probe (5’-AGACTTGAGGCTTTACAAAGTATAG and 3’-TATCATGGATACCCTTCTAAAATTC) hybridized to EcoRV-digested genomic DNA (Fig. 1B), thereby yielding both a 28.5- and an 11-kb fragment (eotaxin-2-deficient and correctly targeted, respectively). Three 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-2 allele to offspring at roughly 50% frequency. High degree chimeric mice were then bred with C57BL/6J mice. Offspring with agouti coat color were genotyped, and mice heterozygote for the eotaxin-2 deletion were bred. Mice homozygous for the eotaxin-2 deletion were compared with wild type littermate controls. To produce eotaxin-2-deficient mice in the pure 129 SvEv background, the high degree chimeric mice were bred to 129 SvEv mice; heterozygote offspring for the eotaxin-2-deficient alleles were mated to produce homozygous eotaxin-2-deficient mice. After the second intranasal challenge. A PCR strategy (5’-GGAATTCCTCGGGG-AGAG and 3’-TTAAAACCCTCGGCTTAATTG primers) was employed to screen mice; PCR of wild-type eotaxin-2 and eotaxin-2-null identified products of 2338 and 3156 bp, respectively (Fig. 1D). To produce inducible IL-13 lung transgenic mice deficient in eotaxin-2, bitransgenic mice (CC10-tTA-IL-13) on an FVB background were bred with eotaxin-2-deficient mice on a 129 SvEv background. F2 mice were genotyped for the eotaxin-2 deletion as well as the CC10 and IL-13 transgenes. Mice that were homozygous null for eotaxin-2 were compared with their littermate control mice that were wild type for eotaxin-2.

OVA-induced Model of Allergic Lung Inflammation—An allergic lung disease mouse model was established by sensitizing mice with an intraperitoneal injection of 100 μg of OVA and 1.0 mg of alum in 200 μl of sterile physiologic saline on two occasions separated by 2 weeks. Two weeks after the last sensitization, two intranasal doses of 50 μg of OVA were given; briefly, mice were aerosolized with isoflurane inhalation and held in a supine position, and 50 μl of normal saline containing 50 μg of OVA was applied to the nares using a micropipette. After the liquid was aspirated into the lung, the mice were held upright until alert. Mice were sacrificed between 18 and 20 h after the second intranasal challenge.

IL-13-induced Airway Responses—An allergic lung inflammation mouse model was established by modifying protocols described previously (49, 50). Mice received three 10-μg doses of IL-13 by intratracheal administration every other day. Mice were anesthetized by an intramuscular injection of ketamine and held in an upright position, and 20 μl of physiologic saline containing 10 μg of recombinant murine IL-13 (a generous gift of Dr. Debra Donaldson, Wyeth Laboratories, Cambridge, MA) was applied directly into the lungs using a micropipette with a gel-loading tip. After instillation, mice were held upright for 20–30 s. Mice were sacrificed between 18 and 20 h after the last challenge.

BALF Analysis—Immediately after being euthanized by CO2 inhalation, a midline neck incision was made, and the trachea was cannulated. The lungs were lavaged twice with 1.0 ml of phosphate-buffered saline. For cellular analysis, the recovered BALF was centrifuged at 400 × g for 5 min at 4°C and resuspended to 0.5 ml of phosphate-buffered saline containing 1% fetal calf serum and 0.5 mM EDTA. Lysis of red blood cells was carried out utilizing RBC lysis buffer (Sigma) according to the manufacturer’s recommendations. Total cell numbers were counted with a hemocytometer. Cytocentrifuge preparations (5 × 106 cells) were stained with Giemsa-Diff-Quick (Dade Diagnostics of Puerto Rico, Inc., Aguada, Puerto Rico), and differential cell counts were determined. For RNA analysis, the lungs were lavaged...
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FIG. 1. Generation of eotaxin-2 gene-targeted mice. A, the endogenous murine eotaxin-2 locus is shown. The position of the three exons, the relevant restriction enzyme sites, and the 3′-probe used to screen targeted ES cells are indicated. The position and genetic organization of a chemokine with homology to human eotaxin-3 are also shown. The predicted nucleotide sequence of the presumed eotaxin-3 cDNA, derived from these exons, has been deposited at GenBank™ with accession number AY587128. B, the position of the homologous recombination arms used to generate the final construct, designed to replace neomycin for exon 1 and exon 2 of eotaxin-2, is shown. The positions of the oligonucleotides used for PCR screening of offspring are also shown. C, genotyping of ES cells and F1 generation pups was done utilizing Southern blot analysis of EcoRV-digested genomic DNA showing an 8.6-kb band created by the eotaxin-2-deficient allele (KO) and a 28.3-kb band created by the wild type allele (WT). D, all F2 and subsequent mice were genotyped utilizing a two-primer PCR strategy taking advantage of the fact that the neomycin (NEO) insert is ~200 bp shorter than the deleted portion of the wild type gene. Restriction enzyme definitions are as follows: RV, EcoRV; RI, EcoRI; B, BamHI; Sm, SmaI.

FIG. 2. IL-13 is required for allergen-induced eotaxin-1 and eotaxin-2 mRNA induction. Northern blot analysis of total lung RNA from wild type (WT) and IL-13 deficient mice (IL-13 KO) that had undergone an OVA model of allergic airway inflammation reveals that eotaxin-1 (Eot-1) and eotaxin-2 (Eot-2) production is IL-13-dependent. RNA was isolated from whole lung 18 h after the second intranasal OVA challenge. 12 μg of RNA was electrophoresed through 1.5% agarose, transferred to hybridization membrane, and probed with sequence-specific probes for eotaxin-1 and eotaxin-2. Each lane represents a separate mouse following saline or OVA treatment. As a loading control the 28 S and 18 S ribosomal RNA following EtBr staining is shown.

Once with 1.0 ml of phosphate-buffered saline. The recovered BALF was immediately centrifuged at 400 × g for 5 min at 4 °C, and the cells were resuspended in TriZol™. RNA was then prepared per the manufacturer's specifications.

Peripheral Blood Eosinophil Analysis—The mice were euthanized by CO2 inhalation. Immediately thereafter, a midline neck incision was used to sever the jugular vein. Peripheral blood was immediately collected with a pipette, and 5 μl was added directly to 45 μl of Discombe’s solution, as reported previously (51).

Quantification of Tissue Eosinophil Levels—After lavage, the left lobe of the lung was removed and fixed with Accustain® neutral buffered 10% formalin (Sigma), embedded in paraffin, cut into 5-μm sections, and fixed to positive charge slides. Eosinophils in the tissue were differentially stained using an antiserum against murine major basic protein (anti-MBP) as described earlier (52). In brief, tissue 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™, Zymed Laboratories Inc.) for 4 min at room temperature, incubated with rabbit anti-murine MBP antibody (1:6000, a kind gift from J. Lee, Mayo Clinic, Scottsdale, AZ) for 1 h at room temperature, and followed by 1:500 dilution of biotinylated goat anti-rabbit IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories) for 30 min each. These slides were further treated with nickel diaminobenzidine-chloride solution to form a black precipitate and were counter-stained with nuclear fast red solution. Quantification of immunoreactive cells was performed by computer-aided image analysis using Image Pro Plus imaging software system (Media Cybernetics, Silver Spring, MD). At least four selections per mouse lung section were analyzed. Using digital image capture, the subepithelial peribronchial tissue regions associated with medium sized bronchioles were quantified for the total MBP+ cell numbers relative to the total tissue area. Calculated eosinophil levels are expressed as cells/mm².

Bone Marrow Eosinophil Colony-forming Unit Analysis—Naïve wild type and eotaxin-2-deficient mice were euthanized by CO2 inhalation, and bone marrow cells were obtained from the femur and centrifuged and resuspended in complete culture RPMI 1640 medium (Invitrogen), 10% fetal calf serum (Invitrogen), 1% penicillin/streptomycin, and 1 M HEPES (Invitrogen). Low density mononuclear cells were separated by density gradient centrifugation over LymphPrep (Accurate Chemicals) for 25 min at 1500 × g at room temperature, and subsequently incubated in plastic flasks for 2 h at 37 °C and with 5% CO2 to remove adherent cells. Nonadherent mononuclear cells (1 × 10⁶) were cultured at 37 °C and with 5% CO2 in 35 × 10-mm tissue culture dishes (Falcon Plastics and Discovery Labware) in 1 ml of methylcellulose culture medium (Methocult™ M3234, Stem Cell Technologies), 20% fetal calf serum and 5 ng/ml of recombinant mouse IL-5 (R&D Systems, Minneapolis, MN). Eosinophil colonies were evaluated after 10 days as described previously (53), according to the criteria established by Lee et al. (54).

In Situ Hybridization of Mouse Lung—In situ hybridization was performed as described (52, 55). In brief, murine eotaxin-1 cDNA in pBS-KS+ plasmid was linearized by restriction enzyme digest with XhoI or EcoRI sense and antisense probes were generated by T7 and T3 RNA polymerase, respectively (RiboProbe Gemini Core System II transcription kit, Promega, Madison, WI). Murine eotaxin-2 cDNA in pBS-KS+ plasmid was linearized by restriction enzyme digest with XhoI or EcoRI sense and antisense probes were generated by T7 and T3 RNA polymerase.
**RESULTS**

**IL-13 Is Required for Eotaxin-1 and Eotaxin-2 Induction**—We were first interested in determining the specific molecules associated with allergen-induced Th2 responses that were responsible for mediating induction of the eotaxins. Whereas IL-13 overexpression has been shown to be sufficient for expression of the eotaxins (27, 28), the requirement of IL-13 for the expression of the eotaxins during the development of allergen-induced lung inflammation has not been addressed. In order to examine this, we subjected IL-13-deficient and matched control mice to OVA-induced experimental asthma. As shown in Fig. 2, Northern blot analysis of lung tissue RNA revealed marked induction of eotaxin-1 and eotaxin-2 in wild type mice but profoundly reduced induction of eotaxin-1 and eotaxin-2 in IL-13-deficient mice (Fig. 2). Thus, IL-13 is required for eotaxin-1 and eotaxin-2 expression in allergic airway inflammation.

**Generation of Eotaxin-2 Gene Targeted Mice**—We were next interested in characterizing the specific role of eotaxin-2 in allergic airway inflammation and aimed to generate eotaxin-2 gene-targeted mice. In order to accomplish this, we took a standard strategy of homologous recombination designed to delete 1.86 kb of DNA corresponding to all the first and second exons of eotaxin-2 and thus producing a null mutation (Fig. 1, A and B). Clones that underwent homologous recombination were screened by Southern blot analysis using the 622-bp 3′-probe as shown in Fig. 1, B and C. Two targeted clones were injected into blastocysts, and both transmitted the disrupted eotaxin-2 allele in the germ line of mice. The eotaxin-2-deficient allele was passed to progeny in Mendelian ratios. For example, of the 169 F2-generation mice genotyped, 47% were heterozygote, 24% were homozygote null, and 28% were wild type mice. We were next interested in verifying that the eotaxin-2 gene-targeting event had indeed eliminated expression of the gene product. In wild type mice, eotaxin-1 is constitutively expressed in a variety of organs; in contrast, the constitutive expression of eotaxin-2 is limited to the spleen, duodenum, and jejunum, suggesting distinct roles for these two chemokines (45). Multitissue Northern blot analysis revealed decreased levels of jejunum eotaxin-2 in mice carrying the heterozygous eotaxin-2 deletion construction (Fig. 3). Most importantly, mice homozygous for the deletion construct had no detectable eotaxin-2 mRNA. Additionally, there was no apparent overexpression of eotaxin-1 to compensate for the loss of eotaxin-2 in the eotaxin-2-deficient mice compared with both wild type and heterozygote littermate control mice.

Finally, we were interested in determining whether the eotaxin-2 gene-targeting event affected the level of eotaxin-1 induction by IL-13. Northern blot analysis of lung RNA from IL-13-treated mice showed that IL-13 treatment induces both eotaxin-1 and eotaxin-2 in the lungs of wild type mice. However, the induction of eotaxin-2 is absent in the eotaxin-2-deficient mice as is eotaxin-1 in the eotaxin-1-deficient mice. Notably, there is no compensatory overexpression of the remaining eotaxin chemokine in either of the gene-targeted mice (Fig. 4).

**Fig. 3.** Eotaxin-1 and eotaxin-2 expression in eotaxin-2 gene-deficient mice. Tissues were collected from naïve F2 generation littermate mice with the following genotype: wild type (eotaxin-2+/+), heterozygous (eotaxin-2+/−), and homozygous null (eotaxin-2−/−). 12 µg of RNA was electrophoresed through 1.5% agarose, transferred to hybridization membrane, and probed with sequence-specific probes for eotaxin-1 (Eot-1) and eotaxin-2 (Eot-2). As a loading control, the 28 S and 18 S ribosomal RNA following EtBr staining is shown.

**Fig. 4.** IL-13-induced expression of eotaxin-1 and eotaxin-2 in the lungs of wild type, eotaxin-1-deficient, and eotaxin-2-deficient mice. Wild type (WT), eotaxin-1 deficient (Eot-1 KO), and eotaxin-2 deficient (Eot-2 KO) mice were treated with repeated doses of IL-13, and the lung RNA was analyzed by Northern blot analysis for eotaxin-1 and eotaxin-2 mRNA expression using sequence-specific probes. 12 µg of RNA was electrophoresed through 1.5% agarose, transferred to a hybridization membrane, and probed with sequence-specific probes for eotaxin-1 and eotaxin-2. Each lane represents a separate mouse following saline or IL-13 treatment, and as a loading control, the 28 S and 18 S ribosomal RNA following EtBr staining is shown. Eotaxin-1- and eotaxin-2-deficient mice were examined along with their respective strain-matched control mice.

**polymerase, respectively (Riboprobe Gemini Core System II transcription kit, Promega, Madison, WI). The radiolabeled α-35S-UTP probes were hybridized and washed under high stringency conditions.**

**AHR Measurements**—AHR was measured using whole body plethysmography, as reported previously (41). Increases in airway resistance to aerosolized methacholine (3.125 to 50 mg/ml) were determined as Penh, a dimensionless value, after 5 min of aerosol exposure.

**Statistical Analysis**—Statistical significance comparing different sets of mice was determined by Wilcoxon rank sum of the median test.
important for CC chemokines (Fig. 5). However, to date we have not been able to demonstrate expression of eotaxin-3 mRNA in the naive, allergen, or IL-13-challenged lung by Northern blot analysis or reverse transcription-PCR using a probe and primers that worked under control conditions (data not shown). Taken together, whereas the eotaxin-2 gene targeting allele is in close proximity to a murine eotaxin-3 homologue, we have no evidence that this is functionally relevant for the purposes of our experiments.

**Eotaxin-2 Gene-targeted Mice Have Normal Base-line Eosinophils**—We were next interested in characterizing whether there was a base-line phenotype in eotaxin-2-deficient mice. Eotaxin-2 homozygous null mice appeared outwardly normal and were indistinguishable from their wild type littermates. To characterize further the role of eotaxin-2 at base line, we examined the levels of eosinophils in the bone marrow, blood, spleen, and jejunum. This analysis revealed no detectable differences between wild type and eotaxin-2-deficient mice in all compartments (Table 1). For comparison, the levels of eosinophils in eotaxin-1-deficient mice (129SvEv) were normal in the blood and spleen but reduced >10-fold in the jejunum (Ref. 50; data not shown). Additionally, eotaxin-2-deficient mice have
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### Table 1

| Tissue             | Wild type | Eotaxin-2 KO |
|--------------------|-----------|--------------|
| Bone marrow (eosinophils/ml × 10^3) | 5.2 ± 2.0 | 7.8 ± 3.4 |
| Spleen (eosinophils × 10^6) | 8.2 ± 4.4 | 7.7 ± 5.3 |
| Jejunum (eosinophils/mm^2) | 366 ± 139 | 358 ± 222 |

Results are from mice on a pure 129 SvEv genetic background and are depicted as the mean ± S.D. For blood, n = 15–18; spleen, n = 13–14; bone marrow, n = 10; and jejunum, n = 8–10 mice.

deficient IL-13 lung transgenic mice had markedly fewer (9-fold decrease) eosinophils in the BALF compared with IL-13 lung transgenic mice containing wild type eotaxin-2 (p < 0.001) (Fig. 6D). Notably, the level of tissue eosinophils was similar for both strains of mice, being 27.6 ± 18.7 and 33.4 ± 22.0 eosinophils/mm^2 for wild type and eotaxin-2-deficient mice, respectively (mean ± S.D., n = 5), consistent with IL-13 delivery. In addition, the tissue distribution of eosinophils was similar in IL-13 lung transgenic mice that contained the wild type eotaxin-2 gene or the eotaxin-2 gene deficiency (Fig. 6E).

To begin to examine the functional consequences of the eotaxin-2 deficiency, we examined AHR in wild type and eotaxin-2-deficient mice (129SvEv) that received intratracheal IL-13. Following three doses of IL-13, wild type and eotaxin-2-deficient mice developed similar degrees of AHR compared with saline-challenged mice. For example, at a methacholine concentration of 12.5 mg/ml, IL-13-treated wild type and eotaxin-2-deficient mice had Penh values of 4.42 ± 0.58 and 3.94 ± 0.46 (mean ± S.E., n = 8 mice), respectively. Following saline treatment, wild type and eotaxin-2-deficient mice had Penh values significantly lower than the IL-13-treated mice, being 1.12 ± 0.46 and 1.57 ± 0.48 (mean ± S.E., p < 0.05, n = 7–6 mice), respectively.

**Similar Induction Patterns for IL-13-induced Eotaxin-1 and Eotaxin-2**—Based on the new data as well as our prior findings that eotaxin-1 selectively regulates IL-13-induced lung tissue eosinophilia (but not IL-13-induced BALF eosinophilia) (39), these collective findings substantiate independent roles for eotaxin-1 and eotaxin-2 in regulating IL-13-induced allergic responses in the lung. Thus, understanding the mechanism of their nonredundant role became the focus of our study. We hypothesized that both eotaxins may have a distinct temporal and/or spatial induction profile. To test this hypothesis, it was important to rule out the possibility that each eotaxin was not overexpressed in IL-13-treated eotaxin-1-deficient and eotaxin-2-deficient mice in order to compensate for the lack of the other chemokine. Northern blot analysis (Fig. 4) revealed no appreciable compensatory overexpression of the remaining chemokine in the specific gene-deficient mice. We were therefore interested in testing the kinetics of eotaxin-1 and eotaxin-2 accumulation following IL-13 delivery to the lung. We took advantage of an inducible IL-13 lung transgenic system because this allows precise regulation of lung IL-13 levels for various times. In these experiments, CC10-rTA-IL-13 mice were exposed to doxycycline for 1–30 days, and the level of eotaxin-1 and eotaxin-2 mRNA was assessed by Northern blot analysis of lung tissue. As shown in Fig. 7, after only 1 day of exposure to doxycycline, both eotaxin chemokines were detected, and peak levels were present by day 10 and reached a plateau between days 10 and 30. Collectively, these results demonstrate that both eotaxin chemokines are induced independently of one another while having similar kinetics of induction in response to IL-13. Thus, the distinct actions of eotaxin-1 and eotaxin-2 in vivo are not likely to be primarily related to the kinetics of their induction.

**IL-13-induced Eotaxin-1 and Eotaxin-2 Are Expressed in a Distinct Spatial Compartment in the Lung**—To gain further mechanistic insight into the distinct roles of eotaxin-1 and eotaxin-2, we were interested in determining whether eotaxin-1 and eotaxin-2 were expressed in different compartments in the lung. The preferential role for eotaxin-2 in regulating IL-13-induced BALF eosinophilia prompted us to compare eotaxin-1 and eotaxin-2 expression in BALF cells compared with the whole lung. As shown in Fig. 8, analysis of whole lung RNA revealed abundant levels of eotaxin-1 and eotaxin-2 mRNA in the doxycycline-induced IL-13 lung transgenic mice. However, in marked contrast to analysis of the normal eotaxin colony-forming units in the bone marrow having 7.6 ± 3.2 eotaxin colonies/ml compared with 7.4 ± 1.8 in wild type mice (mean ± S.D., n = 5).

**Eotaxin-2-deficient Mice Have Decreased IL-13-induced Airway Eosinophilia**—We next examined the role of eotaxin-2 in the recruitment of eotaxin-1 into the lung. Eotaxin-2-deficient and wild type control mice were intratracheally administered IL-13 or saline. We chose to deliver three doses of 10 μg of IL-13, because that dose has been shown to be optimal for eosinophil recruitment into the lung (56). Eotaxin-2 levels were subsequently determined in the peripheral blood, BALF, and lung tissue 18 h after the last dose of IL-13. There were no significant differences between IL-13-treated wild type and eotaxin-2-deficient mice in the peripheral blood or the peribronchial region of the lung tissue (Fig. 6, A and B). However, there was a remarkable decrease in the recruitment of eosinophils into the BALF of eotaxin-2-deficient mice in response to IL-13 (Fig. 6C). For example, the BALF of IL-13-treated wild type mice had 1.3 ± 1.7 × 10^4 eosinophils; in contrast, eotaxin-2-deficient mice had an ~50-fold decrease in IL-13-induced BALF eosinophilia (2.2 ± 2.6 × 10^3 cells) (p < 0.001). These data were corroborated in eotaxin-2-deficient mice that were in the pure 129 SvEv genetic background, wherein the peripheral blood of IL-13-treated mice had 1.1 ± 0.3 × 10^5 and 1.0 ± 0.8 × 10^5 (mean ± S.D., n = 9) for wild type and eotaxin-2-deficient mice, respectively, and the peribronchial regions had 12.8 ± 14.4 × 10^5 and 26.5 ± 19.2 × 10^5 (mean ± S.D., n = 7) for wild type and eotaxin-2-deficient mice, respectively. Furthermore, eotaxin-2-deficient mice (on a pure 129 SvEv genetic background) had 1.4 ± 1.9 × 10^6 eosinophils in the BALF, whereas wild type control mice had 8.4 ± 9.6 × 10^6 eosinophils (mean ± S.D., n = 9) (p < 0.02). We also examined the effect of eotaxin-2 deficiency on the recruitment of non-eosinophils into the BALF in response to IL-13. This analysis revealed no significant differences between macrophage, neutrophil, or lymphocyte populations in the BALF of IL-13-treated wild type and eotaxin-2-deficient mice, although there was a trend for decreased levels of neutrophils and lymphocytes. For example, levels of BALF macrophages, lymphocytes, and neutrophils increased by IL-13 from 2.5 ± 1.7 × 10^4 to 8.7 ± 10^5, 0.8 ± 1.8 × 10^5 to 8.0 ± 21 × 10^4, and 1.2 ± 1.1 × 10^5 to 1.3 ± 1.6 × 10^5 cells/mouse, respectively, in wild type mice (mean ± S.D., n = 7 mice) and for saline and 13 mice for IL-13-treated mice). In eotaxin-2-deficient mice, levels of BALF macrophages, lymphocytes, and neutrophils increased from 2.3 ± 1.2 × 10^4 to 4.1 ± 3.3 × 10^4, 2.8 ± 5.5 × 10^4 to 5.9 ± 13 × 10^4, and 9.2 ± 4.1 × 10^4 to 1.9 ± 1.7 × 10^5 cells/mouse, respectively (mean ± S.D., n = 8 mice for saline and 19 mice for IL-13-treated mice). The specific role of eotaxin-2 in regulating airway eosinophilia was likely to be due mainly to a local effect in the lung because IL-13-treated mice had similar levels of eosinophils in the blood of wild type and eotaxin-2-deficient mice (Fig. 6A).

To examine the role of eotaxin-2 in a different system, we generated inducible IL-13 lung transgenic mice genetically deficient in eotaxin-2. After 10 days on doxycycline, eotaxin-2-deficient mice had markedly fewer (9-fold decrease) eosinophils in the BALF compared with IL-13 lung transgenic mice containing wild type eotaxin-2 (p < 0.001) (Fig. 6D). Notably, the level of tissue eosinophils was similar for both strains of mice, being 27.6 ± 18.7 and 33.4 ± 22.0 eosinophils/mm^2 for wild type and eotaxin-2-deficient mice, respectively (mean ± S.D., n = 5), consistent with IL-13 delivery. In addition, the tissue distribution of eosinophils was similar in IL-13 lung transgenic mice that contained the wild type eotaxin-2 gene or the eotaxin-2 gene deficiency (Fig. 6E).
whole lung, the BALF cells selectively expressed only eotaxin-2 mRNA. These results suggest that the preferential expression of eotaxin-2 by luminal inflammatory cells provides a likely mechanism to explain the ability of eotaxin-2 to selectively regulate eosinophil recruitment into the airway lumen.

IL-13-induced Eotaxin-2 Is Expressed by Macrophages in the Airway—Based on the striking difference in the location of eotaxin-1 and eotaxin-2 expression, we were interested in determining the exact cellular source of eotaxin-2 in the allergen-challenged murine lung. In order to examine this, we performed in situ hybridization for eotaxin-2 mRNA expression. Hybridization of the eotaxin-2 antisense riboprobe to CC10-rtTA-IL-13 mouse lung samples after 10 days on doxycycline revealed strong staining in individual cells in the tissue and airway (Fig. 9, A and B). High power magnification of the eotaxin-2 mRNA+ cells revealed that staining was present in macrophages in the tissue and airway lumen, as shown by the representative light-field photomicrograph (Fig. 9C).

**DISCUSSION**

Recent attention concerning the pathogenesis of allergic lung disease has focused on understanding the role of IL-13 because it has been identified as an important regulator of multiple aspects of experimental disease including AHR, eosinophilia,
and mucus overproduction (16, 26, 57, 58). Although IL-13 has been shown to be a powerful inducer of multiple chemokines and antagonism of IL-13 diminishes OVA-induced lung eosinophilia (26, 27, 59), the mechanism of IL-13-induced lung eosinophilia has not been established (57, 58). In the present study we have employed a mouse model system to dissect the contribution that IL-13 has in regulating eotaxin production and the specific role of eotaxin-2 in regulating IL-13-induced eosinophilia in the lung. To accomplish this goal, we have generated eotaxin-2-deficient mice, and we report the effect of IL-13 administration in these mice. First, our results establish that IL-13 is an essential mediator of allergen-induced eotaxins in experimental asthma as demonstrated by the almost complete loss of eotaxin-1 and eotaxin-2 expression in asthmatic IL-13-deficient mice. Second, our results establish that the expression of IL-13-induced eotaxin-1 and eotaxin-2 occurs independently of each other, as demonstrated by the normal induction patterns of eotaxin-1 in eotaxin-2-deficient mice and of eotaxin-2 in eotaxin-1-deficient mice. Most interestingly, we did not find induction of a gene homologous to human eotaxin-3, which we found at a 7-kb downstream from eotaxin-2 on murine chromosome 5. Consistent with a lack of induction of the putative eotaxin-3 mRNA with allergen challenge, the presumed promoter regions (2 kb of genomic DNA located 5′ upstream of the ATG of exon 1) lack a consensus signal for signal transducers and activators of transcription binding, as is typically seen with other functional eotaxin promoters (60, 61). Thus, a true mouse homolog for human eotaxin-3 does not exist, because even if the gene can be expressed under some circumstances, they are not the same circumstances of expression found in the human system. It is also interesting to note that the current public expression sequence tag (EST) database does not contain cDNA sequences corresponding to “murine eotaxin-3,” suggesting that this may represent a pseudogene. Determining if this region contains a real chemokine gene and the function of the putative coding protein are currently under investigation. Third, we have established that IL-13-induced lung eosinophilia is differentially regulated by eotaxin-2 compared with eotaxin-1. In particular, our prior studies have shown that eotaxin-1 is required for the development of peribronchial eosinophilia (39). Our current study, demonstrating that eotaxin-2 is critical for the development of eosinophilia in the airway lumen (in response to IL-13), provides an integrated mechanism to explain the nonredundant roles for these two selective CCR3 ligands. The normal level and the distribution of lung tissue eosinophils observed in eotaxin-2-deficient mice raise the possibility that eotaxin-2 may have a direct effect on luminal eosinophils. The differential regulation of eosinophil trafficking within the lung by eotaxin-1 and eotaxin-2 begins to elucidate the level of control the immune system has at its disposal to recruit an effector cell (here an eosinophil) to a specific target area (the peri-bronchial lung tissue-eotaxin-1 or the airway lumen-eotaxin-2) by utilizing only one chemokine receptor (CCR3). Fourth, our results provide a mechanistic understanding for the molecular basis of the nonredundant role of eotaxin-1 and eotaxin-2 in IL-13-driven lung responses. In particular, we demonstrate that both chemokines are expressed in predominantly different compartments in the lung; eotaxin-1 is expressed by the lung tissue, and indeed prior studies have shown eotaxin-1 to be primarily made by macrophages and eosinophils (62, 63), and eotaxin-2 is expressed primarily by airway lumen macrophages. These results are in agreement with human bronchial biopsy studies from asthmatics that have demonstrated an increase in the number of eotaxin-1-producing cells in the bronchial epithelium and inflammatory cells (62, 64). Indeed, these studies are also in agreement with the finding that human peripheral blood monocytes can be induced to make large quantities of eotaxin-2 following exposure to IL-4 (65).

Here we report the first general characteristics of novel eotaxin-2-deficient mice. Consistent with results seen in eotaxin-1 (37), eotaxin-2-deficient mice have no gross developmental disorders and yield offspring at rates predicted by Mendelian inheritance. It is interesting to note that eotaxin-2-deficient mice have no gross alterations in the base-line levels of eosinophils in the hematopoietic and gastrointestinal tissues. This is important for several reasons. First, even though human eotaxin-2 was originally identified as a hematopoietic progenitor cell inhibitory cytokine (43), our results suggest that the primary function of the murine homologue is not related to hematopoiesis at least under base-line conditions. Second, in marked contrast to eotaxin-1-deficient mice, eotaxin-2-deficient mice do not have a base-line deficiency in jejunal eosinophils. This is consistent with the finding that eotaxin-1 has a relatively high constitutive expression pattern compared with eotaxin-2 (Fig. 3) (45). This finding highlights the relatively unique and nonoverlapping role that eotaxin-1 has in regulating eosinophil base-line homing.

In our study, the level of IL-13-mediated eosinophil accumulation was low compared with antigen-driven allergic inflammatory responses (experimental asthma models) (37, 66). This may reflect the requirement for the up-regulation of both the eotaxins and IL-5 and their synergistic interactions for maximal eosinophilic responses. In particular, IL-5 promotes an expansion of circulating eosinophils in response to antigen provocation, whereas eotaxin subsequently sequesters eosinophils into tissues (15, 67). Additionally, IL-5 has been shown to induce CCR3 on eosinophil precursor cells (68). Although our preliminary studies of IL-13-induced AHR revealed that...
eotaxin-2 was not required for this functional response, examination of AHR induced by antigen-driven allergic responses will be of interest.

In summary, our results have established the following: 1) a critical role for IL-13 in allergen-induced expression of the eotaxins; 2) a critical role for eotaxin-2 in mediating IL-13 induced airway eosinophilia; and 3) a mechanistic understanding of the nonredundant roles of eotaxin-1 and eotaxin-2 in mediating IL-13-induced lung responses involving differential regional expression of eotaxin-2 (primary in the airway lumen macrophages) and eotaxin-1 (primarily in the lung tissue). Taken together, these results identify a linear relationship in which allergen induces IL-13, which in turn is required for eotaxin-2 induction, which in turn regulates airway eosinophilia in a manner distinct from eotaxin-1 (Fig. 10). These results combined with prior reports that IL-5 and eotaxins can indirectly regulate IL-13 levels (41) further demonstrate the integrative and cooperative signaling mechanisms in allergic lung responses.

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