Murine Eotaxin-2: A Constitutive Eosinophil Chemokine Induced by Allergen Challenge and IL-4 Overexpression[1]

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The generation of tissue eosinophilia is governed in part by chemokines; initial investigation has identified three chemokines in the human genome with eosinophil selectivity, referred to as eotaxin-1, -2, and -3. Elucidation of the role of these chemokines is dependent in part upon analysis of murine homologues; however, only one murine homologue, eotaxin-1, has been identified. We now report the characterization of the murine eotaxin-2 cDNA, gene and protein. The eotaxin-2 cDNA contains an open reading frame that encodes for a 119-amino acid protein. The mature protein, which is predicted to contain 93 amino acids, is most homologous to human eotaxin-2 (59.1% identity), but is only 38.9% identical with murine eotaxin-1. Northern blot analysis reveals three predominant mRNA species and highest constitutive expression in the jejunum and spleen. Additionally, allergen challenge in the lung with *Asperigillus fumigatus* or OVA revealed marked induction of eotaxin-2 mRNA. Furthermore, eotaxin-2 mRNA was strongly induced by both transgenic over-expression of IL-4 in the lung and administration of intranasal IL-4. Analysis of eotaxin-2 mRNA expression in mice transgenic for IL-4 but genetically deficient in STAT-6 revealed that the IL-4-induced expression was STAT-6 dependent. Recombinant eotaxin-2 protein induced dose-dependent chemotactic responses on murine eosinophils at concentrations between 1–1000 ng/ml, whereas no activity was displayed on murine macrophages or neutrophils. Functional analysis of recombinant protein variants revealed a critical role for the amino terminus. Thus, murine eotaxin-2 is a constitutively expressed eosinophil chemokine likely to be involved in homeostatic, allergen-induced, and IL-4-associated immune responses. *The Journal of Immunology*, **2000, 165: 5839–5846.**

Eosinophil accumulation in the peripheral blood and tissues is a hallmark feature of several important medical diseases including atopic disorders (allergic rhinitis, asthma, and eczema), parasitic infections, and numerous systemic diseases (e.g., Churg-Strauss syndrome, eosinophilic pneumonia, eosinophilic gastroenteritis, and the idiopathic hypereosinophilic syndrome; Ref. 1). The finding that eosinophils normally account for only a small percentage of circulating or tissue-dwelling cells and that their numbers markedly and selectively increase under specific disease states indicates the existence of molecular mechanisms that regulate the selective generation and accumulation of these leukocytes. The pathological role of eosinophils primarily occurs in tissues; therefore, a major focus of scientific investigation on eosinophils has been to elucidate the processes involved in eosinophil tissue recruitment. Numerous mediators have been identified as eosinophil chemoattractants, including diverse molecules such as lipid mediators (platelet activating factor, leukotrienes), bacterial products (fMLP), and recently chemokines such as RANTES and macrophage inflammatory protein (MIP)[3]-1α (2). However, none of these mediators selectively promote eosinophil recruitment. In contrast, a group of human chemokines, designated eotaxins, with eosinophil-selective chemoattractant activity has been identified.

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. Using an in vivo chemotaxis assay in guinea pig skin, the partial amino acid sequence for the protein responsible for eosinophil chemoattraction in the bronchoalveolar fluid in allergen-challenged guinea pigs was determined (3). This facilitated the genetic cloning of the genes and cDNA for guinea pig, murine, and human eotaxin, and the identification of eotaxin as a member of the CC chemokine family most homologous to the macrophage chemoattractant protein (MCP) subfamily (4–7). This subfamily of eotaxin and MCP chemokines is clustered on human chromosome 17q11, a region that also contains other CC chemokines (such as MIP-1α, I-309, RANTES, and hemofiltrate CC chemokine (HCC)-1 and -2, Ref. 8). Interestingly, this region has been recently linked to asthma susceptibility (9). Using genomic analyses, rather than biological assays, two additional chemokines have been identified in the human genome that encode for CC chemokines with eosinophil-selective chemoattractant activity, and have hence been designated eotaxin-2 and eotaxin-3 (10–13). Eotaxin-2 and eotaxin-3 are only distantly related to eotaxin-1 because they are only ~30% identical in sequence and are located in a different chromosomal position

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[3] Abbreviations used in this paper: MIP, macrophage inflammatory protein; EST, expressed sequence tag; MCP, macrophage chemoattractant protein; ORF, open reading frame; UTR, untranslated region.

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(7q11.23). The specific activity of all three human eotaxin chemokines is mediated by the selective expression of the eotaxin receptor, CCR3, a seven-transmembrane-spanning G protein linked genetically polymorphic receptor, primarily expressed on eosinophils (14–17). CCR3 is a promiscuous receptor; it interacts with multiple ligands including MCP-2, -3, -4, RANTES, and HCC-2 (MIP-5, leukotactin); however, the only ligands that signal exclusively through this receptor are the eotaxin chemokines, accounting for the cellular selectivity of eotaxin. Interestingly, other cells involved in allergic responses, Th2 cells and basophils, also express CCR3 (18–20); however, the significance of CCR3 expression on these cells has been less clearly demonstrated than on eosinophils.

A variety of approaches have been used to determine the biological role of the eotaxin chemokines. Eotaxin-1 is induced by allergen challenge in the human respiratory tract and eotaxin-1 and eotaxin-2 are over-expressed in allergic inflammatory tissue (21–23). However, the relative contribution of the eotaxin chemokines compared with other chemoattractants has not been extensively addressed. Substantial progress has been made by analysis of the role of chemokines in mice. In particular, Ab neutralization experiments and eotaxin-1 gene targeting in mice have revealed a nonredundant role for eotaxin-1 in allergic lung inflammation and in the regulation of eosinophil homing to the gastrointestinal tract (24–27). Eotaxin-1 gene-targeted mice have a marked deficiency of tissue-dwelling eosinophils at baseline (25) and this has recently facilitated the identification of a role for eosinophils in postnatal mammary gland development (28). Although eotaxin-1 gene-targeted mice have a marked deficiency in tissue-dwelling eosinophils, they still have resident eosinophils, especially in the gastrointestinal tract when IL-5 is overexpressed in vivo (29). Understanding the full biological and pathological role of eosinophils will likely be facilitated by identification and characterization of the eotaxin-2 and eotaxin-3 homologues in mice. We now report the characterization of murine eotaxin-2 cDNA and protein.

Materials and Methods

Identification of murine eotaxin-2 cDNA

The nucleotide sequence of human eotaxin-2 (CC chemokine ligand (CCL)-24 according to new classification (30)) was used to screen the public expressed sequence tag (EST) database by use of BLASTN searches. The cDNA clones AI536479 and AI099007, encoding a putative murine homologue of eotaxin-2, were obtained from Genome Systems (St. Louis, MO) and subjected to sequencing. The predicted cleavage site of the mature form of eotaxin-2 was determined by use of the SignalP server at http://www.cbs.dtu.dk/services/SignalP/.

Genomic DNA analysis

The eotaxin-2 gene was amplified by PCR from genomic DNA isolated from a wild-type mouse. The primers used were located just upstream of the ATG (5′-CTG TGC CTG ACC TCC AGA AC-3′) and at the end of the open reading frame (ORF) (5′-CTA AAC CTC GGT GCT ATT GC-3′). The 2.2-kb PCR product was subcloned into pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced. Location of exons was determined by homology with the cDNA sequence. All splice donor and acceptor sites conform with the established consensus.

RNA preparation and Northern blot analysis

RNA from multiple tissues (stomach, jejunum, liver, kidney, spleen, lung, thymus, brain, and testis) was isolated from BALB/c mice using the Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA was electrophoresed in an agarose-formaldehyde gel, transferred to Gene Screen transfer membranes (NEN, Boston, MA) in 10× SSC, and cross-linked by UV radiation. The murine eotaxin-2 cDNA fragment was isolated from clone AI099007 with *Eco*RI and *Not*I digestion; cDNA fragments were radiolabeled with ^{32}P using the Klenow reaction with random priming. Membranes were hybridized and washed under standard conditions and exposed to film for 3–7 days.

IL-4 overexpression

Wild-type or STAT-6 gene-targeted mice (31) expressing the murine IL-4 transgene under the control of lung-specific Clara cell 10 promoter (32) were generated by mating F_1 crosses of IL-4-transgenic mice and STAT-6 gene-targeted mice with STAT-6 gene-targeted mice. The IL-4 transgene and the STAT-6 gene-targeted locus were screened by PCR analysis (31, 32) and RNA was isolated from whole lungs. In other experiments, ether-anesthetized wild-type mice were treated with 2 μg intranasal rIL-4 (PeproTech, Rocky Hill, NJ) complexed to 10 μg anti-IL-4 mAb (BVD4-1D11 (33); a gift from DNAX Research Institute, Palo Alto, CA) in 20 μl of saline on three occasions separated by 2 days. Following 24 h after the last dose, whole lung RNA was isolated.

Murine models of asthma

A mouse model of allergic lung disease was established using methods described previously (29, 34). In brief, mice were lightly anesthetized with Metofane inhalation (methoxy-fluorane; Pittman-Moore, Mundelein, IL) and 100 μg (50 μl) *Asperigillus fumigatus* (Bayer Pharmaceuticals, Spokane, WA) or 50 μl of normal saline alone was applied to the nasal cavity using a micropipette with the mouse held in the supine position. After instillation, mice were held upright until alert. After three treatments per week for 3 wk, mice were sacrificed between 18 and 20 h after the last intranasal challenge. Alternatively, mice were sensitized and challenged with OVA as previously described (24).

Eotaxin protein production

Recombinant murine eotaxin-2 protein was produced by PeproTech in *Escherichia coli* from a recombinant strain carrying a synthetic gene sequence encoding eotaxin-2 preceded with a synthetic leader sequence, MKKKSLAMVTIP. The leader sequence is underlined and was removed by cleavage with aminopeptidase, an enzyme that that cleaves the amino acid penultimate to proline, therefore yielding eotaxin-2 (IP). Alternatively, the leader sequence was removed by treatment with cyanogen bromide that cleaves following a methionine, thus yielding eotaxin-2 (VTIP). The protein was purified by sequential column chromatography and the sequence and/or purity was verified by mass spectroscopy, Edman sequencing, HPLC, and SDS-PAGE. Additionally, eotaxin-2 (IDSVTIP) was purchased from R&D Systems (Minneapolis, MN). A comparable eotaxin-2 amount in the three preparations was shown by SDS-PAGE separation followed by Coomassie staining. Recombinant murine eotaxin-1, KC and MIP-1α were obtained from PeproTech.

Chemotaxis assay

Chemotactic responses were determined by transmigration through either bare filters (macrophages) or respiratory epithelial cells (granulocytes) as previously described (35). In brief, A549 cells (American Type Culture Collection, Manassas, VA) were grown as monolayers in tissue-culture flasks in DMEM (Life Technologies) supplemented with 10% FCS, penicillin, and streptomycin. Cell monolayers were trypsinized, centrifuged, and resuspended in fresh medium before culture on permeable filters (polycarbonate filters with 3-μm pores) in Transwell tissue-culture plates (Corning Costar, Cambridge, MA). Cells (1.5×10^5) in 100-μl volumes were grown to confluence on the upper surface of the filters for 2 days and treated with 10 ng/ml TNF-α for 18 h. Leukocytes (1×10^6) in HBSS plus 0.5% BSA (low endotoxin; Sigma, St. Louis, MO) were placed in the upper chamber and the chemokine (in HBSS plus 0.5% BSA) was placed in the lower chamber. Eosinophils were obtained by use of splenocytes from IL-5-transgenic mice (4); neutrophils were obtained from the peritoneal cavity 3 and 18 h after injection of 9% (w/v) casein (Sigma). Transmigration was allowed to proceed for 3 h for neutrophils and 2.5 h for splenocytes. Macrophages were obtained from the peritoneal cavity of mice 72 h after injection of 3% (w/v) thioglycollate (Becton Dickinson, Mountain View, CA). Cells were placed in the upper chamber of the bare filters with 5-μm pores and allowed to migrate toward the chemokines placed in the bottom chamber for 3 h. Finally, cells in the lower chamber were counted in a hemacytometer, cytocentrifuged, stained by immersion for 2 min in Harleco Wright-Giemsa stain (EM Science, Gibbstown, NJ) followed by DiffQuick (Fisher Scientific, Pittsburgh, PA) and the differential white cell analysis was determined microscopically.

Results

Characterization of murine eotaxin-2 cDNA and gene

The human eotaxin-2 and eotaxin-3 cDNA sequences were used to screen the public mouse EST databank. Interestingly, over the course of this study, two murine ESTs that were homologous to

human eotaxin-2, but no eotaxin-3 homologues, have been identified. This suggests that the mouse genome may in fact contain only two eotaxin genes. The mouse eotaxin-2 homologues were derived from mammary gland cDNA libraries. An additional EST derived from porcine small intestine was also homologous to eotaxin-2 (70% identity over 385 bp). The two mouse EST clones (accession numbers AI536479 and AI099007) were subjected to sequencing and the consensus sequence of the ORF was found to have considerable homology to human eotaxin-2 (58%); homology to other chemokines was significantly less (e.g., 35.1 and 35.6% homology to murine eotaxin-1 and human eotaxin-3, respectively). Using the EST sequences, the longest 5′ untranslated region (UTR) was found to contain 53 bp, whereas the 3′ UTR contained 183 bp. The nucleotide sequence of the consensus of the two clones verified by sequencing is shown in Fig. 1*A*. To elucidate the genomic organization of the eotaxin-2 gene, genomic DNA was amplified by PCR using primers immediately upstream of the start codon and at the end of the ORF. A 2.2-kb fragment was sequenced and compared with the cDNA sequence. This analysis demonstrated that the DNA encoding eotaxin-2 ORF is located on three exons (Fig. 1*B*). The three exons contain 73, 118, and 169 bp of the ORF, respectively (Fig. 1*A*). The introns are 0.2 and 1.6 kb in size. This is comparable to the human eotaxin-2 gene, which can be located under accession number AC005102 (the complete sequence of a bacterial artificial chromsome containing human chromosome 7q11.23-q21.1 including eotaxin-2 and eotaxin-3 genes). The cDNA contains an ORF that encodes for a protein of 119 amino acids (Fig. 2). The deduced polypeptide sequence contains a highly hydrophobic amino-terminal region characteristic of a signal peptide with a predicted cleavage site between Ser[26] and Val[27]. The predicted mature protein of 93 amino acids has a molecular mass of 10,335 Da and an isoelectric point of 10.4. There are two potential sites for *N*-glycosylation at positions 54 and 115 and one for *O*-glycosylation at threonine in position 28. The mature protein is a CC chemokine with four cysteine residues in the proper arrangement and is most homologous to human eotaxin-2 (59.1%) and only 38.9 and 38.2% homologous to murine eotaxin-1 and human eotaxin-3, respectively. An alignment of murine and human eotaxin-2 (Fig. 2) reveals an equal number of amino acids and comparable conservation of homology throughout the protein sequence (including the leader sequence) and conservation of the amino-terminal valine in the mature protein.

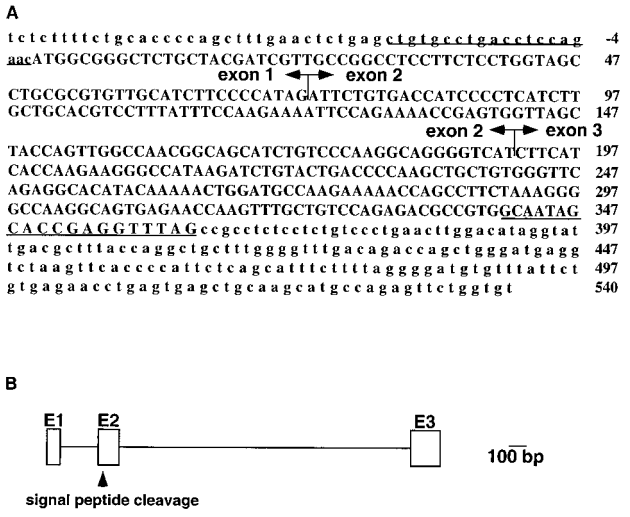

FIGURE 1. Sequence of murine eotaxin-2. *A*, The consensus sequence of the two murine eotaxin-2 ESTs (AI536479 and AI099007) from the public data bank as confirmed by sequencing is shown. Bases encoding for protein are shown in capital letters; bases in UTR are shown in small letters. Numbering is based on +1 being the first base of the ORF. The primers used for PCR amplification of the genomic segment are underlined. Exon-intron boundaries are indicated. This sequence has been deposited to GenBank under accession number AF281075. *B*, A schematic representation of the genomic organization is shown. Exons are depicted as boxes and introns as lines. The location of the signal peptide cleavage site is shown.

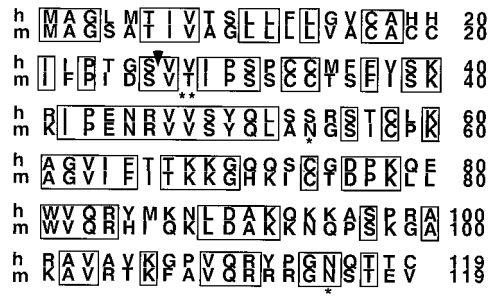

FIGURE 2. Alignment of murine and human eotaxin-2. The boxed amino acids are identical between the two proteins. The signal sequence cleavage site is indicated with an arrow. ∗, *N*-linked glycosylation sites. ∗∗, *O*-linked glycosylation site.

Eotaxin-2 mRNA expression pattern

Northern blot analysis of multiple organs from healthy mice revealed the presence of readily detectable eotaxin-2 mRNA in the jejunum and spleen and to a lesser extent in the liver and lung (Fig. 3). The following three bands were evident: 0.8, 1.1, and 1.8 kb. Interestingly, while the 0.8-kb species was of the highest intensity in the spleen; the 0.8- and 1.1-kb species were of equal intensity in the jejunum. No specific hybridization was noted in the kidney, thymus, brain, or testis. In comparison, eotaxin-1 was expressed more ubiquitously, with readily detectable expression in the stomach, jejunum, lung, and thymus (Fig. 3).

Eotaxin-2 mRNA expression is induced by allergen challenge

Eotaxin-1 was originally described as an allergen-induced gene product in the rodent lung and has subsequently been shown to be directly induced by allergen challenge in humans (36). However, although human eotaxin-2 has been shown to be up-regulated in allergic inflammatory tissue (23) and allergen-induced late phase

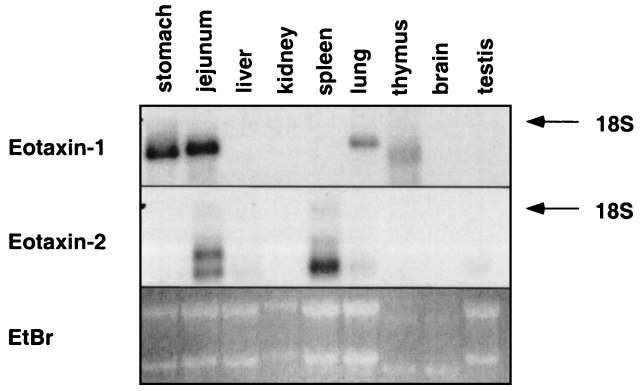

FIGURE 3. Distribution of eotaxin-2 mRNA. Total RNA (10 μg) obtained from mouse organs from BALB/c mice was electrophoresed, transferred, and hybridized with radiolabeled eotaxin-1 and eotaxin-2 cDNA probes. Autoradiography was performed for 7 days for eotaxin-2 and 4 days for eotaxin-1. The location of 18S RNA is shown on the *right* and the cDNA probe is indicated on the *left*. Ethidium bromide (EtBr) staining of the RNA gel is also shown.

responses in the skin (37), the ability of allergen to directly induce this gene product in the lung has not been determined. Therefore, we were interested in determining whether eotaxin-2 mRNA was induced in the lung in response to allergen challenge using a mouse model of asthma. We subjected mice to repeated challenges with intranasal *A. fumigatus* allergen; these conditions induce eotaxin-1 expression (Fig. 4) and promote eosinophil recruitment into the lung. This allergen challenge protocol was found to induce a marked increase in the level of eotaxin-2 mRNA compared with saline challenged mice (Fig. 4). To demonstrate that eotaxin-2 induction was not specific to the *A. fumigatus* allergen, OVA-sensitized mice were subjected to intranasal challenge with OVA. Eotaxin-2 mRNA was also increased by this allergen (data not shown).

Eotaxin-2 expression is regulated by IL-4 and STAT-6

We were next interested in determining whether murine eotaxin-2 is induced by IL-4 because this cytokine is critically involved in the pathogenesis of allergic lung disease (38). In addition, IL-4 has been shown to induce murine eotaxin-1 and human eotaxin-1–3 (4, 12, 39, 40). To assess this, we examined the lungs of IL-4 lung-transgenic mice. Interestingly, these mice had markedly increased levels of eotaxin-2 mRNA compared with wild-type mice (Fig. 5). Similar to eotaxin-2, eotaxin-1 mRNA was also induced by the IL-4 transgene. However, RANTES mRNA was not modulated by IL-4 expression. To confirm the ability of IL-4 over-expression in the lungs to induce eotaxin production, wild-type mice were treated with intranasal IL-4 directly. Administration of IL-4 was also found to induce eotaxin-1 and eotaxin-2 mRNA in the lungs and this effect was dose-dependent (data not shown).

The down-stream effects of IL-4 are mediated by at least two distinct pathways, one involving STAT-6 and the other involving insulin receptor substrates-1/2 (38). Therefore, we were interested in determining which signaling pathway was involved in IL-4-induced eotaxin production. To examine this, we generated wild-type and STAT-6-deficient mice that were also transgenic for IL-4. Analysis of eotaxin-1 and eotaxin-2 mRNA expression in these mice revealed that the IL-4-induced expression of these chemokines was completely dependent upon STAT-6 (Fig. 5). In contrast, the baseline expression of RANTES was not dependent on STAT-6.

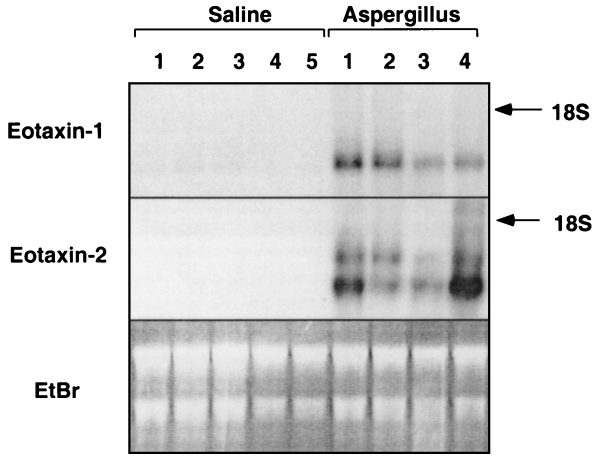

FIGURE 4. Eotaxin-2 mRNA expression is induced by allergen challenge. Total RNA from the lungs of FVB mice treated with saline or *A. fumigatus* was hybridized with radiolabeled eotaxin-1 and eotaxin-2 cDNA probes. Each lane represents a separate mouse. Autoradiography was performed for 3 days. The location of 18S RNA is shown on the *right* and the cDNA probe is indicated on the *left*. Ethidium bromide (EtBr) staining of the RNA gel is also shown.

Eotaxin-2 is an eosinophil-specific chemokine

We were next interested in determining the chemoattractant activity of the murine eotaxin-2 protein. We first examined this by analysis of leukocyte transmigration in response to a concentration gradient of eotaxin-2. Because wild-type mice do not have appreciable numbers of eosinophils, splenocytes were isolated from IL-5-transgenic mice. Although splenocytes from these mice contain a mixed population of cells (30–40% eosinophils), the only cells that were able to transmigrate in response to eotaxin-2 were eosinophils (Fig. 6*A* and data not shown). For example, murine eosinophils exhibited a strong chemotactic response to murine eotaxin-2. A dose response was seen between doses of 1 and 10 ng/ml with a plateau demonstrated at doses of 10–1000 ng/ml (Fig. 6*A*). In contrast, eotaxin-1 exhibited a bell-shaped dose response with a peak visible at 10 ng/ml and a decrease in chemotactic activity at higher doses (Fig. 6*B*). The experiments in Fig. 6, *A* and *B*, were performed on separate occasions; when eotaxin-1 and -2 were compared in the same experiment, the efficacy and potency of the two chemokines was not significantly different (paired *t* test, $p = 0.32$, $n = 3$). Checkerboard analysis with varying concentrations of eotaxin-2 in the upper and lower chambers revealed only chemotaxis without any chemokinesis (data not shown). High doses of chemokines have recently been proposed to induce reverse chemotaxis (41); however, at doses up to 1 or 5 μg/ml of eotaxin-2 or eotaxin-1, respectively, no reverse chemotaxis was induced (data not shown). To test the selectivity of eotaxin-2, chemotactic responses were also determined for purified neutrophils (Fig. 6*C*) and macrophages (Fig. 6*D*). Recombinant eotaxin-2 (at doses up to 1000 ng/ml) had no activity on these cell types even though they responded to control chemokines MIP-1α and KC.

Structure-function analysis of the amino terminus of eotaxin-2

It has been suggested that ligand-induced activation of the eotaxin receptor is dependent upon the chemokine's amino terminus (8). Therefore, we were interested in determining the functional role of the amino terminus of eotaxin-2. Interestingly, sequence analysis of the recombinant eotaxin-2 protein indicated that the first two amino-terminal amino acids were deleted compared with the predicted mature protein (based on the consensus sequence for signal peptide cleavage). Therefore, it was of interest to also examine the activity of eotaxin-2 with a mature amino terminus. We subsequently produced a small-scale preparation of eotaxin-2 containing an amino terminus starting with the amino-acid sequence VTIP, and compared its function to the original preparation of eotaxin-2 (IP). Additionally, during the final preparation of this manuscript, recombinant murine eotaxin-2 became commercially available (from R&D Systems); this protein preparation contained three additional amino acids on the amino terminus, designated eotaxin-2 (IDSVTIP). We subsequently examined the activity of all three preparations of eotaxin-2 by testing their ability to induce eosinophil transmigration. Eotaxin-2 (VTIP) reached a plateau at lower concentrations (1 ng/ml) than eotaxin-2 (IP) (10 ng/ml) indicating higher potency (paired *t* test, $p = 0.04$, $n = 3$; Fig. 7); however, the maximal chemotactic response was not significantly different between both forms of eotaxin-2 (paired *t* test, $p = 0.78$, $n = 3$). Interestingly, the amino-terminally extended eotaxin-2 (IDS VTIP), displayed no chemotactic activity at doses up to 100 ng/ml and only weak chemotactic activity at 1000 ng/ml (Fig. 7). At a higher dose (10,000 ng/ml) of this preparation of eotaxin-2 (IDSVTIP), chemotactic activity was comparable to 1 ng/ml

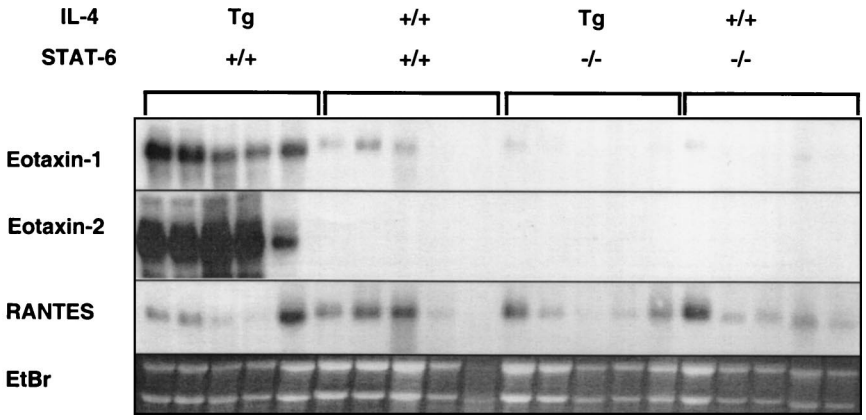

FIGURE 5. Eotaxin-2 expression is regulated by IL-4 and STAT-6. Total RNA from the lungs of IL-4-transgenic (Tg) or wild-type (+/+) mice carrying the wild-type (+/+) or targeted disruption of the STAT-6 gene (−/−) was hybridized with the radiolabeled probes indicated. Ethidium bromide (EtBr) staining of the RNA gel is also shown.

eotaxin-2 (VTIP) and 10 ng/ml eotaxin-2 (IP) (data not shown). These data indicate that the rank order of potency of the different eotaxin-2 variants is VTIP > IP > IDSVTIP.

Discussion

Although three chemokines with eosinophil-selective activity have been described in the human genome (designated eotaxin-1, eotaxin-2, and eotaxin-3), only one chemokine with eosinophil selectivity has been described in the mouse genome. Interestingly, all three human eotaxins selectively interact with the same receptor, designated the eotaxin receptor (CCR3), but these CC chemokines are only ~30% homologous to each other. This structural diversity provided an important distinction that allowed designation of the presently described eosinophil-selective CC chemokine, which is 59% homologous to human eotaxin-2 (but only ~38% homologous to eotaxin-1 or eotaxin-3), as murine eotaxin-2. Identical with human eotaxin-2 (10), murine eotaxin-2 contains 119 amino acids including a predicted leader sequence with 26 amino acids. In comparison, eotaxin-1 (murine and human) and eotaxin-3 are smaller chemokines with 94 and 97 amino acids, respectively, and both contain a 23 amino acid leader sequence (3, 4, 12, 13). Of note, similar to the genes for all three human eotaxins (13, 42), murine eotaxin-2 consists of a three-exon gene. Analysis of the positions of the intron/exon sites reveals identical locations between murine and human eotaxin-2, further substantiating that these genes are homologues.

Experimental analysis of the role of chemokines in homeostatic processes and immunity has been facilitated by studies in rodents. This has been particularly useful for analysis of chemokine-induced eosinophil trafficking because eotaxin-1 was originally discovered using a guinea pig model of allergic lung inflammation and subsequently shown to have conserved structure and function in mice and humans. For example, eotaxin-1 in mice and man has been demonstrated to be an allergen-induced gene product in the respiratory tract where expression directly correlates with eosinophil tissue recruitment, and neutralization of its activity diminishes eosinophil chemoattraction in vitro and in vivo (26, 36). The eotaxin-1 gene-targeted mice have an impairment in eosinophil recruitment into the respiratory tract following allergen challenge (24) and a marked deficiency of eosinophils throughout the gastrointestinal tract at baseline and following oral allergen challenge (29, 43). However, although tissue eosinophils are reduced in eotaxin-1-deficient mice and in mice depleted of eotaxin by Ab neutralization, these mice still have residual tissue eosinophils. In particular, mice over-expressing IL-5 in the absence of the eotaxin-1 gene, have substantial levels of eosinophils in the jejunum compared with wild-type mice (29). Taken together, these results indicate the importance of eotaxin-1-dependent and -independent pathways in the regulation of eosinophil tissue recruitment. In the present study, murine eotaxin-2 is demonstrated to have shared and distinct features compared with eotaxin-1. In particular, both genes are shown to be allergen-induced and increased by IL-4 via a STAT-6-dependent pathway in the lung. Additionally, while both chemokines are constitutively expressed, their tissue expression pattern is distinct. Eotaxin-1 appears to be more ubiquitously expressed in the gastrointestinal tract (Fig. 3) (29) compared with eotaxin-2, which is predominantly expressed in the jejunum. Furthermore, eotaxin-1 is expressed in the thymus (Fig. 3) where it regulates eosinophil trafficking to the medulla (25). Thus, eotaxin-1 and eotaxin-2 are likely to coregulate eosinophil trafficking during homeostatic, allergen-induced, and IL-4-associated responses; however, their tissue-specific and temporal roles are likely to be distinct.

To demonstrate direct eosinophil chemotactic activity, we have isolated eosinophils from CD2-driven IL-5-transgenic mice and measured eosinophil chemotaxis using a transmigration assay in vitro. Despite the large numbers of eosinophils present in the hematopoietic organs of these mice, there are few tissue eosinophils except in the gastrointestinal tract (29, 44). This benign phenotype suggests that other signals (in addition to IL-5) are necessary for eosinophil tissue recruitment and activation. By using eosinophils from these mice, we have shown that eotaxin-2 is a potent chemoattractant for eosinophils. By combining the results of comparative experiments between eotaxin-1 and eotaxin-2, both chemokines are demonstrated to have comparable activity. Whereas eotaxin-1 displays a typical bell-shaped curve with a peak activity at 10 ng/ml, eotaxin-2 reaches a plateau at 10 ng/ml and does not have diminished activity at doses as high as 1000 ng/ml. It is likely that the eosinophils isolated from IL-5-transgenic mice have been primed in vivo by IL-5 exposure and that this potentiates their responsiveness to both eotaxin-1 and eotaxin-2. Consistent with this, IL-5 activates human eosinophils and primes them to respond to RANTES and eotaxin-1 in vitro (45–47). We have also examined the chemoattractant activity of eotaxin-2 on neutrophils and macrophages in vitro and observed no activity under conditions where control chemokines induced chemoattraction. Although we have not ruled out the possibility that eotaxin-2 interacts with macrophages, neutrophils, or other cells under other conditions, these results have consistently shown that eotaxin-2 is a strong eosinophil chemoattractant relative to other cell types.

The results presented also give insight into the structure-function of eotaxin-2. The predicted amino-terminal amino acids of mature murine eotaxin-2 start with VTIP. Consistent with this, amino-acid sequence analysis of human eotaxin-2 produced in Sf9 insect cells infected with a recombinant baculovirus has revealed that the human protein begins with VVIP, indicating conservation

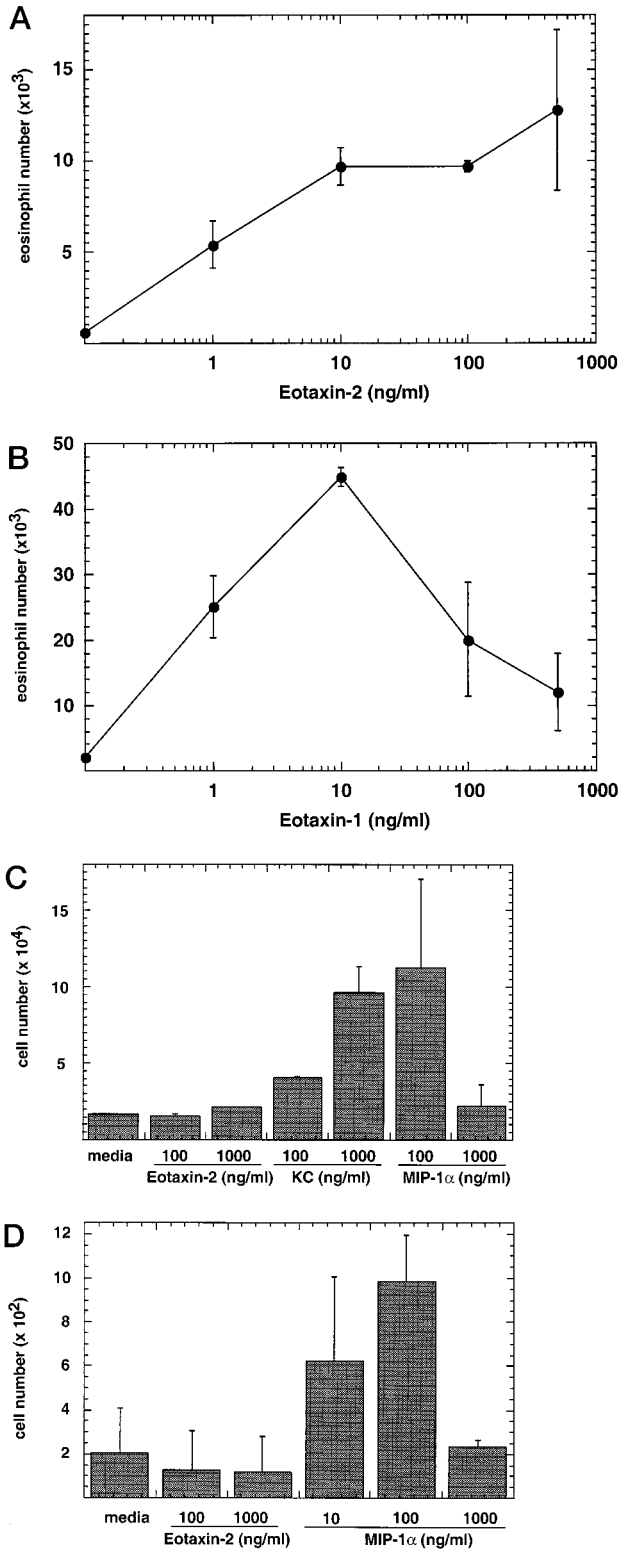

FIGURE 6. Eotaxin-2 chemotaxis assay. Splenocytes from IL-5-transgenic mice were allowed to transmigrate through a layer of respiratory epithelial cells in response to the indicated doses of eotaxin-2 (*A*) and eotaxin-1 (*B*). Following a 2.5-h incubation, cells in the bottom chamber were counted in hemacytometer, and cytocentrifuge slides were used for differential cell counts. Each data point was performed in duplicate and represents mean ± SD. A representative experiment for each of the chemokines is shown ($n = 4$). *C*, Peritoneal neutrophils were subjected to transmigration in response to the indicated doses of eotaxin-2, KC, and MIP-1α. Following a 3-h incubation, cells in the bottom chamber were counted by hemacytometry, and cytocentrifuge slides were used for differential cell counts. Data are represented as the total number of cells in the bottom well; staining of the cells revealed that >80% were neutrophils. *D*, Peritoneal macrophages were subjected to chemotaxis over bare filters in response to the indicated doses of eotaxin-2 and MIP-1α. Following a 3-h incubation, cells in the bottom chamber were counted by hemacytometry, and cytocentrifuge slides were used for differential cell counts.

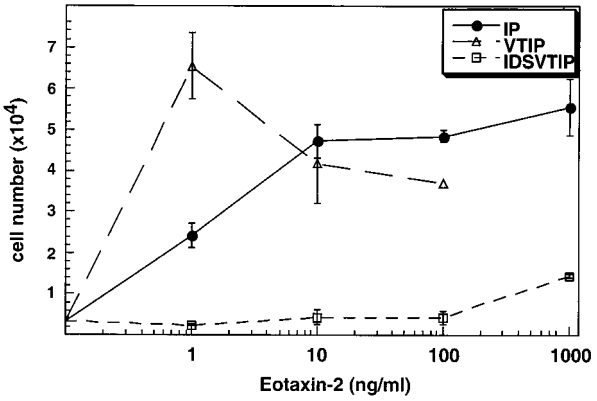

FIGURE 7. Comparison of eotaxin-2 preparations with different amino-terminal amino acids. Three different preparations of eotaxin-2 were used in an eosinophil transmigration assay. The preparations are labeled according to the sequence of the amino terminus (IP, VTIP, and IDSVTIP). Cells were allowed to transmigrate through a layer of respiratory epithelial cells in response to the indicated doses of eotaxin-2. Following a 2.5-h incubation, cells in the bottom chamber were counted in hemacytometer, and cytocentrifuge slides were used for differential cell counts. Each data point was performed in duplicate and represents mean ± SD. A representative experiment is shown ($n = 3$).

with the predicted site in the mouse protein. Interestingly, our initial preparation of murine eotaxin-2 produced in bacteria started with IP because the VT was removed during purification by the aminopeptidase cleavage. In other experiments, we were able to produce eotaxin-2 with an intact native amino terminus (starting with VTIP). Interestingly, this protein was more potent displaying a reduced ED_{50}, compared with the amino-terminally truncated eotaxin-2. Finally, recently murine eotaxin-2 became commercially available; however, the commercial source indicated that their preparation of murine eotaxin-2 contained three additional amino-terminal amino acids (IDS-VTIP) compared with the predicted mature protein. Interestingly, this preparation of eotaxin-2 was considerably less potent (>1000-fold) than either of the other two eotaxin preparations. Thus, the amino terminus of murine eotaxin-2 is involved in interacting with its receptor. The amino terminus of numerous chemokines has been demonstrated to be critically involved in triggering receptor activation (48). For example, removal of the first two amino acids of mature human eotaxin-1 by CD26, a dipeptidyl peptidase, results in markedly reduced eosinophil chemoattractive activity (49).

In summary, we have characterized the sequence, expression pattern, and gene organization of murine eotaxin-2 (CC chemokine ligand-24). Our results have several biological messages concerning eosinophils and chemokines. First, the mouse is demonstrated to contain two functional eotaxin chemokines, indicating that analysis of eosinophil trafficking in experimental murine models needs to account for both of these activities. Second, a differential pattern of constitutive expression of eotaxin-1 and eotaxin-2 is demonstrated, suggesting usage of distinct homeostatic chemokines by

eosinophils in different tissues. Third, eotaxin-2 expression is increased in response to allergen challenge and transgenic overexpression of IL-4 in the lung. This response is similar to eotaxin-1, however, distinct from RANTES, another eosinophil-active CCR3 ligand. Fourth, STAT-6 is demonstrated to be required for the induction of eotaxin-1 and eotaxin-2 expression by chronic IL-4 stimulation. This is the first study to show that chemokine expression induced by continuous signaling through the IL-4 receptor remains dependent upon STAT-6. Last, we demonstrate the functional importance of the amino terminus of eotaxin-2. It is hoped that further analysis of this murine chemokine will contribute to understanding the broad functional role of chemokines and eosinophils.

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