

In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration

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Abstract: Eosinophil migration from circulation is controlled, in part, by chemokines through a family of G-protein-coupled chemokine receptors (CCR). Studies of human eosinophils have demonstrated that signaling through CCR3 receptors is a prominent pathway leading to chemotaxis, although several other receptor-ligand interactions also appear to mediate eosinophil recruitment. The availability of genetically unique strains of mice permits a reductionist approach to assess the signaling pathways in experimental models of human disease. However, despite similarities in these pathways between mice and humans, significant species differences exist, complicating the translation of results from animal models to humans. Purified mouse eosinophils were used in this study to investigate the chemokine receptor expression and the activities of 18 chemokines. Mouse eosinophils isolated from IL-5 transgenic mice expressed transcripts encoding the chemokine receptors CCR1, CCR2, CCR3, CCR5, CCR8, CXCR2, and CXCR4, but not CCR4. Mouse eosinophils also migrated in response to human and mouse eotaxin-1 and -2, but not human eotaxin-3. In addition, the induced migration of mouse eosinophils by TARC, MIP-1 β , and KC suggests that unidentified receptor-ligand interactions contribute to eosinophil recruitment. It is interesting that the potent chemoattractant of human eosinophils, RANTES, was unable to mediate mouse eosinophil migration. Furthermore, despite the ability of MIP-1 α to bind receptors on purified mouse eosinophils, it was only able to induce significant eosinophil migration in a mixed splenocyte population and was unable to induce migration of highly purified eosinophils. Collectively, these observations reveal physiologically relevant distinctions in mechanisms mediating human and mouse eosinophil migration that potentially reflect evolutionary disparities between these species. *J. Leukoc. Biol.* 71: 1033–1041; 2002.

Key Words: eotaxin · TARC · KC · MIP-1 · RANTES

INTRODUCTION

Eosinophils are granulocytic leukocytes that selectively accumulate in tissues in a variety of disease states, including helminth infestations and allergic inflammatory responses (e.g., asthma and allergic dermatitis, rhinitis, and conjunctivitis). These bone marrow-derived cells are released into peripheral blood before migrating into tissues where they exert effector functions through multiple, often concurrent, mechanisms [1]. The signals and mechanisms controlling the chemoattractant-mediated migration to sites of inflammation depend, in part, on dynamic interactions between adhesion molecules expressed on eosinophils as well as the vascular endothelium. In addition, the generation of chemoattractive signaling gradients within target tissues appears to be critical for the selective movement of eosinophils [2].

The use of mouse models to dissect pathways of allergic inflammation and determine the roles of individual cell types and chemokines/chemokine receptors in allergic pathophysiology has increased dramatically. The ability of these models to develop antigen-induced recruitment of lymphocytes and eosinophils [3], increase antigen-specific immunoglobulins, and increase T-helper cell type 2 cytokines and chemokines [4–6], coupled with the ease of genetic manipulation in these animals (for example, see ref [7]), has led to the widespread use of mice as models of human allergic inflammation. The expanded use of mouse models has thus necessitated a more thorough examination of chemokine-eosinophil interactions in mice in order to gain a better understanding of how these responses may differ from those responses in humans.

Chemokines are a family of small peptides (8–14 kD) produced by many cells involved in allergic inflammation, including endothelial cells [8, 9], epithelial cells [10], fibroblasts [11], smooth muscle [12, 13], monocytes/macrophages [14], T cells [15], mast cells [16], and eosinophils [15]. Chemokines act by binding to cells through G-protein-coupled seven-transmembrane receptors on leukocytes, which promote an array of

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cellular events associated with vectorial movement including integrin activation, cytoskeletal reorganization, degranulation, and the generation of oxygen radicals and bioactive lipids [17]. Human eosinophils have been shown to express the chemokine receptors CCR1 [18], CCR3 [18, 19], CXCR2 [20], and CXCR4 [21]. In turn, several chemokines have been shown to elicit the migration of human eosinophils through several CC-chemokine receptors including eotaxin-1 [22], -2 [23], and -3 [24]; monocyte-chemotactic protein (MCP)-2 [25], -3 [26], and -4 [27]; regulated on activation, normal T cell expressed and secreted (RANTES) [28]; macrophage inflammatory protein (MIP)-1 α [29]; interleukin (IL)-8 [20]; and macrophage-derived chemokine (MDC) [30]. In contrast, mouse eosinophils have only been shown to express CCR1 and CCR3 [31]. Accordingly, only mouse eotaxin-1 [32] and -2 [33] (a mouse orthologue of eotaxin-3 has not been identified to date) and MIP-1 α [31] have been shown to have effects on mouse eosinophil migration.

Nominal amounts of data characterizing mouse eosinophil migration in response to chemokines exist as a result of logistical constraints associated with the availability of peripheral blood eosinophils in sufficient numbers and purity for experimental manipulation. As a result, the available data are not comprehensive and are found as part of several independent studies, making it difficult to assess the relative potencies of the chemokines examined. In this study, IL-5 transgenic mice were used to generate pure (>98%) populations of peripheral blood eosinophils to investigate the ability of several chemokines to induce migration *in vitro*. These data demonstrate that although mouse eosinophils express CCR1 and CCR3, only responses to CCR3 agonists were significant using purified eosinophils. CCR1 ligands MIP-1 α and RANTES were unable to elicit migration of purified eosinophils. Chemokines presumed to bind to CCR4/CCR8 [m thymus and activation-regulated chemokine (TARC) and m MIP-1 β] and CXCR2 (m KC) also demonstrated abilities to elicit purified eosinophil migration. Moreover, the response to TARC was accompanied by an increase in intracellular calcium flux, suggesting the presence of an additional chemokine receptor on mouse eosinophils.

MATERIALS AND METHODS

Reagents

All studies were performed with chemokines purchased from R&D Systems (Minneapolis, MN) with the exception of Figures 3A and 4, in which the mouse eotaxins 1 and 2 were obtained from PeproTech (Rocky Hill, NJ). ¹²⁵I-radiolabeled MIP-1 α and eotaxin were purchased from Perkin Elmer Life Sciences (Boston, MA). HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]), EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis[β -aminoethyl ether]tetraacetic acid), digitonin, Percoll, and glucose were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR). Fetal calf serum (FCS), Hank's balanced salt solution (HBSS), and Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies (Rockville, MD). Anti-CD45 and -CD90 antibody conjugated magnetic beads were obtained from Miltenyi Biotec (Auburn, CA).

Mice

IL-5 transgenic mice, line NJ.1638, were generated as described previously [34]. All procedures were conducted on specific pathogen-free mice 4–6 months of age that were maintained in ventilated microisolator cages housed in an AAALAC-accredited animal facility. Protocols and studies involving animals were conducted in accordance with National Institutes of Health and Mayo Clinic Foundation guidelines.

Isolation of mouse eosinophils

We have developed methods to isolate eosinophils to greater than 98% purity from the IL-5-expressing transgenic line NJ.1638 [34]. Constitutive expression of IL-5 in these mice leads to a marked increase in circulating total white blood cells as well as a compositional shift to nearly 60% eosinophils. As a consequence, greater than 5×10^8 eosinophils can be recovered from 2 ml blood. Cells were routinely obtained from NJ.1638 mice by collecting 300–400 μ l tail blood from each of four mice into 5 ml PBS containing 2% heparin. The blood was then layered onto a single-step Percoll gradient [60% Percoll ($\rho=1.084$), $1 \times$ HBSS, 15 mM HEPES (pH 7.4)] and was centrifuged (45 min, 2000 *g*, 4°C). The buffy coat containing lymphocytes and eosinophils was removed and washed twice in PBS containing 2% FCS. The cell pellet was then subject to brief hypotonic lysis in order to disrupt any contaminating red blood cells, then washed and resuspended in PBS containing 2% FCS. Eosinophils were subsequently isolated by removing the contaminating lymphocytes using a magnetic cell-separation system (Miltenyi Biotec), according to the manufacturer's recommendations. Briefly, B cells and T cells were removed by positive selection following incubation with antibody conjugated magnetic beads specific for CD45-R (B220) and CD90 (Thy 1.2), which bind B cells and T cells, respectively. Recovered cells were washed twice, resuspended in RPMI 1640, and maintained at a concentration of 1×10^7 cells/ml at 4°C. The purity of the recovered cells was determined by visual examination of Wright's stained cytospin preparations. Cell viability was determined each day by trypan blue exclusion.

Splenocyte isolation

Splenocytes were isolated from nontransgenic littermates. Briefly, spleens were removed and placed in RPMI-1640 media containing 2% FCS. Single cell suspensions were obtained by passing a suspension of splenocytes repeatedly through a 22-gauge needle and 40 μ m filter. Viability of the cells was >95% as determined by trypan blue exclusion.

Receptor-binding assays

Binding of ¹²⁵I-labeled chemokines (typically a total of 2×10^4 cpm) to intact mouse eosinophils (typically 5×10^5) at 31°C for ¹²⁵I-eotaxin, or room temperature for ¹²⁵I-labeled MIP-1 α , was performed in the presence of varying concentrations of unlabeled ligands essentially as described [18].

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of chemokine receptor RNA

Total RNA was isolated from purified eosinophils using Trizol reagent (Gibco Life Technologies) according to the manufacturer's protocol. Purified RNA was stored at -80°C until reverse transcribed. Reverse transcription and the generation of PCR amplicons representing individual mouse chemokine receptors (CCR1, CCR2, CCR3, CCR4, CCR5, CCR8, CXCR2, and CXCR4) were performed as described previously by Fischer et al. [35]. Total RNA that was not reverse transcribed served as the negative control in the PCR amplification. PCR products were visualized on a 1.2% agarose gel following staining with ethidium bromide.

Transmigration assay

The migration of purified eosinophils or splenocytes in response to various chemokines *in vitro* was investigated using 5 μ m polycarbonate membrane Transwell inserts in 24-well tissue-culture polystyrene plates (Costar, Corning, NY). The inserts were preincubated with media (RPMI 1640 containing 5% FCS) for 1 h. Media was removed, and cells (1×10^6 ; purified eosinophils included 30 ng/ml recombinant mouse IL-5), in a total volume of 200 μ l media,

were placed into the inserts. The inserts were then placed into the wells containing 500 μ l media alone or media containing chemokines. The plates were incubated at 37°C and 5% CO₂ for 90 min. Assay conditions including cell number, FCS, and IL-5 concentrations and incubation time were optimized using mouse eotaxin-1 and mouse RANTES.

The number of cells that had migrated in these Transwell assays was determined as the sum of the cells in the lower chamber plus the cells that had migrated but remained attached to the bottom of the Transwell insert. Cells that had migrated through the insert and were in the lower chamber were collected into a microcentrifuge tube. The cells that had migrated to the lower chamber, but remained attached to the insert, were recovered by initially removing the cells in the upper chamber, as well as any remaining suspension (by wiping with a cotton-tipped applicator). The attached cells were then recovered by placing the bottom of the inserts into 500 μ l ice-cold PIPES buffer containing 5 mM EDTA and tapping the plates lightly. The cells displaced from the membrane were added to the corresponding microcentrifuge tubes containing lower chamber cells and were counted using a Coulter particle counter (Model Z1, Beckman/Coulter, Fullerton, CA). Differential counts for splenocyte migration were determined by counting Wright's stained cytospin slides (Cytospin 3, Shandon Scientific, Pittsburg, PA) counting ≥ 300 cells. Cell migration is expressed as a migration index, which is the ratio of the number of cells migrating in response to chemokine relative to the number of cells migrating in response to media alone.

Intracellular calcium measurements

Eosinophils (1×10^6 cells/ml) were loaded with 2 μ M Fura-2 in RPMI 1640 containing 2% FCS and were incubated in the dark for 1 h at 37°C. Cells were washed twice in assay buffer (HBSS containing 1 mM CaCl₂), resuspended at 1×10^6 cells/ml, and used within 1 h. The cytoplasmic-free Ca²⁺ concentration of continually stirring cells ($2 \times 10^6/2$ ml) in response to various chemokines was measured using a Hitachi F-4500 spectrofluorometer with excitation at 340 nm and 380 nm and emission at 510 nm. Data are presented as a ratio of fluorescence at 340 nm and 380 nm. Maximal and minimal values were obtained by lysing the cells with 20 μ M digitonin and chelation with 10 mM EGTA, respectively.

Alignment and phylogenetic analysis

Nucleotide and amino acid sequences were obtained from Genbank using published accession numbers. Amino acid sequences were aligned using CLUSTAL with a gap penalty of 5.0 [36]. In addition, Blossum and Pam and Gonnet amino acid weight matrices were used in the different alignments. Alignments with the various amino acid weight matrices did not differ drastically, so we report only those results using the Blossum weighting matrix. Once the amino acid alignments were obtained, the nucleic acid sequences were adjusted to reflect the insertion of gaps in the amino acid alignments [37]. Because the gaps that were introduced into the nucleic acid sequences cover more than a single base, we recorded these gapped regions as single characters as in DeSalle and Brower [38]. Mouse B-lymphocyte chemoattractant was used as an out-group gene to polarize the relationships of the chemokines comprising the remaining in-group genes. The presented phylogenetic analyses were performed with the aligned sequences using a gap penalty of 5. Trees derived from alignments using a wide range of gap penalties (5, 25, 50, 100) were relatively consistent. The nucleic acid characters and the recorded gaps were analyzed together using a single matrix and jackknife resampling analysis, with 1000 sampling replicates, to assess the robustness of the phylogenetic analysis using the program PAUP [39].

Statistical analysis

Data presented are the means \pm SE. Statistical analysis was performed on parametric data using *t*-tests with differences between the means considered significant when *P* < 0.05.

RESULTS

Isolation and viability of eosinophils from IL-5 transgenic mice

Eosinophils isolated from the peripheral blood of IL-5 transgenic mice exhibit the morphology of mature, terminally dif-

ferentiated metamyelocytes. The nuclei are heterochromatic, predominantly ring-shaped, and appear occasionally as "figure 8"-like structures. The cytoplasm contains a preponderance of eosin-staining secondary granules and had no basophilic staining properties. The purity of isolated eosinophils was greater than 98% [contaminating cells included neutrophils (1%) and monocytes (0.5%)], and the cells lost little to no viability for more than 72 h after isolation when maintained at 4°C (Fig. 1A). In vitro Transwell migration in response to eotaxin was also not significantly decreased for at least 72 h following isolation (Fig. 1B).

Chemokine receptor expression on mouse eosinophils

RT-PCR was used to examine chemokine receptor gene expression in purified mouse eosinophils. mRNA encoding the mouse chemokine receptors CCR1, CCR2, CCR3, CCR5, CCR8, CXCR2, and CXCR4 was detected, whereas CCR4 mRNA was undetectable (Fig. 2A). Equilibrium-binding studies performed on purified mouse eosinophils using MIP-1 α and eotaxin-1 demonstrate that receptors for these chemokines are expressed on these cells and bind their cognate ligands with an approximate equal affinity ($IC_{50} \approx 1$ nM; Fig. 2B).

Mouse eosinophils migrate in response to chemokines predominantly through CCR3 receptors

The ability of known CCR1 and CCR3 ligands to mediate eosinophil migration was assessed using m eotaxin-1, m eotaxin-2, m RANTES, and m MIP-1 α in a transmigration assay. Mouse eotaxin-2 was the most potent chemokine examined, reaching a >17-fold increase in maximal response at 30 nM (Fig. 3A). [We observed that the mouse eotaxin-2 obtained from R&D Systems had minimal activity compared with mouse eotaxin-2 obtained from PeproTech. Therefore, the studies that make a quantitative assessment of eotaxin potency (Figs. 3A and 4) were conducted with the PeproTech chemokines.] Sur-

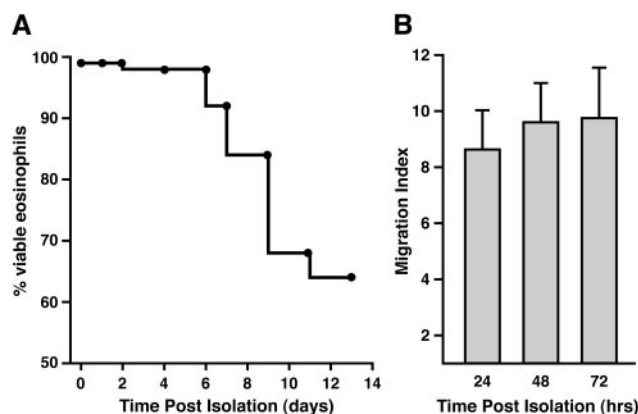


Fig. 1. Eosinophils isolated from IL-5 transgenic mice are viable and responsive for several days. (A) Purified eosinophils (>98%) were maintained in RPMI 1640 at 4°C, and viability was determined on the indicated days by trypan blue exclusion. Values presented are representative of three separate determinations. (B) Eosinophil migration in response to 10 nM mouse eotaxin-1 was assessed for 72 h following cell isolation. Values presented are means \pm SE of five determinations conducted in duplicate.

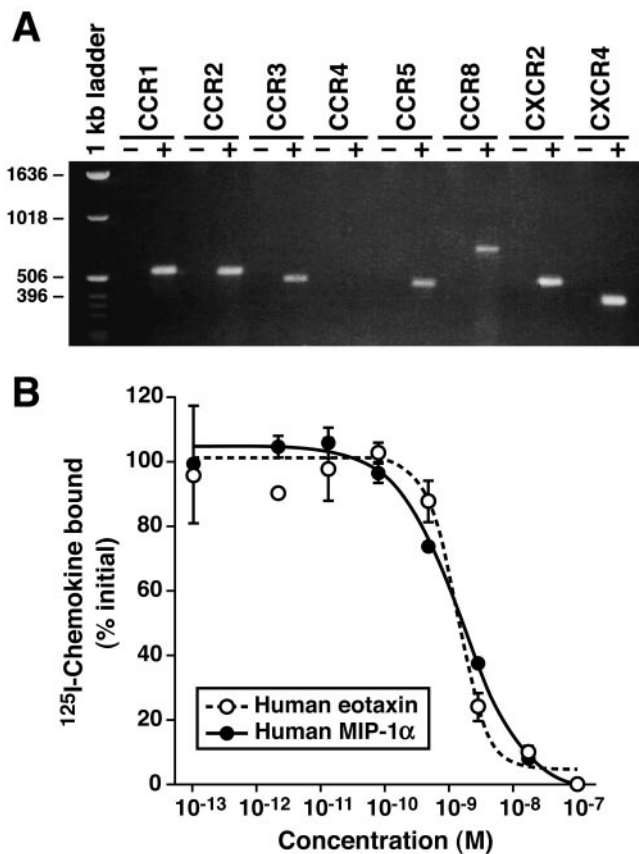
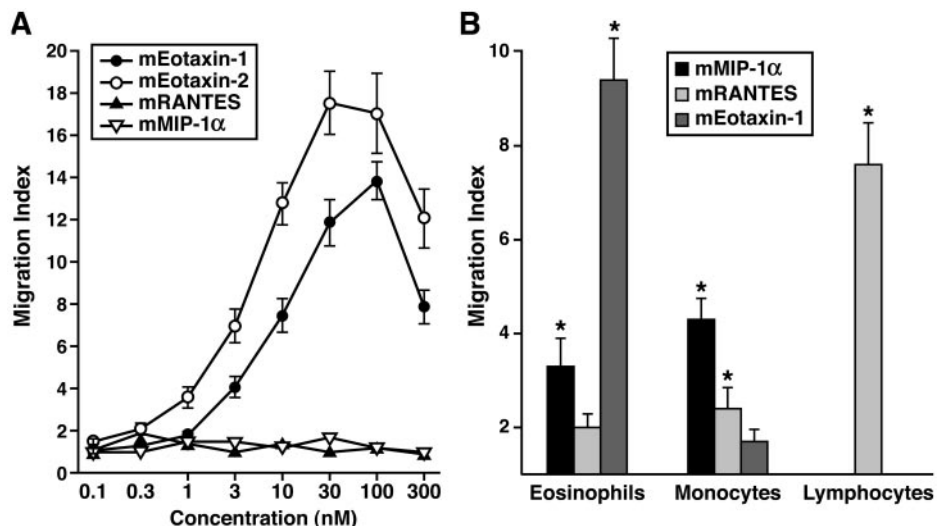


Fig. 2. Multiple chemokine receptors are expressed on eosinophils derived from IL-5 transgenic mice. (A) RT-PCR amplification of mouse eosinophil RNA reveals the expression of several CC and CXC chemokine receptors. (–) Non-RT controls. (B) Competitive ligand-binding studies were performed on purified eosinophils. Leukocytes were incubated with ¹²⁵I-labeled human eotaxin or human MIP-1α and increasing concentrations of unlabeled eotaxin-1 or MIP-1α. Values presented are means ± SE of duplicate determinations conducted in triplicate.

prisingly, the CCR1 agonists m MIP-1α and m RANTES had no significant effects on the migration of purified eosinophils at any concentration (0.1–300 nM). However, in contrast to the studies using purified eosinophils, m MIP-1α demonstrated a

Fig. 3 Purified mouse eosinophils migrate in response to mouse eotaxins but not mouse RANTES or MIP-1α. (A) Purified mouse eosinophil and (B) mouse splenocyte migration in response to these CCR1- and CCR3-binding chemokines were assessed using a Transwell insert assay system, and the data are expressed as a migration index (see Materials and Methods). Values presented are means ± SE of duplicate determinations conducted on three separate occasions. *, Significantly different (*P*<0.05) from baseline.



nominal effect on eosinophil migration in studies using a mixed population of leukocytes derived from the spleen (Fig. 3B). In comparison, regardless of the leukocyte population used (i.e., pure eosinophils or mixed leukocytes), RANTES failed to elicit eosinophil migration (Fig. 3). Significantly, MIP-1α and RANTES induced significant migration of specific mononuclear subpopulations, demonstrating the functionality of these chemokines in this assay system.

Non-CCR3 binding chemokines induce eosinophil migration

Several additional chemokine ligands shown to act through other receptors were similarly tested. Eighteen chemokines originally were screened at concentrations of 30 nM and 100 nM. The rank order of potencies was identical at both doses, and thus the results at 100 nM were arbitrarily chosen and are shown in Figure 4. Chemokines thought to bind to CCR3 (eotaxins and human MCP-4), with the exception of human eotaxin-3, showed the greatest responses in the transmigration assay. Moreover, only three other chemokines (m TARC, m MIP-1β, and m KC) showed significant activity (more than twofold). The baseline migration for all assays presented was consistently approximately 1% of total cells.

TARC induces a calcium flux in purified mouse eosinophils

The ability of m TARC, m MIP-1β, and m KC to bind and signal specifically through eosinophil cell surface receptors was assessed indirectly by measuring intracellular calcium changes following exposure to these chemokines. Mouse eotaxin-1, mouse eotaxin-2, and m TARC caused immediate and significant increases in eosinophil intracellular calcium levels (Fig. 5). In contrast, m MIP-1β and m KC failed to induce a significant calcium flux. Similar results were observed at 24, 48, and 72 h post isolation for all chemokines examined.

Sequence identity and evolutionary relationship between chemokines

Sequences derived from a representative sample of the expanded family of mouse and human chemokines were sub-

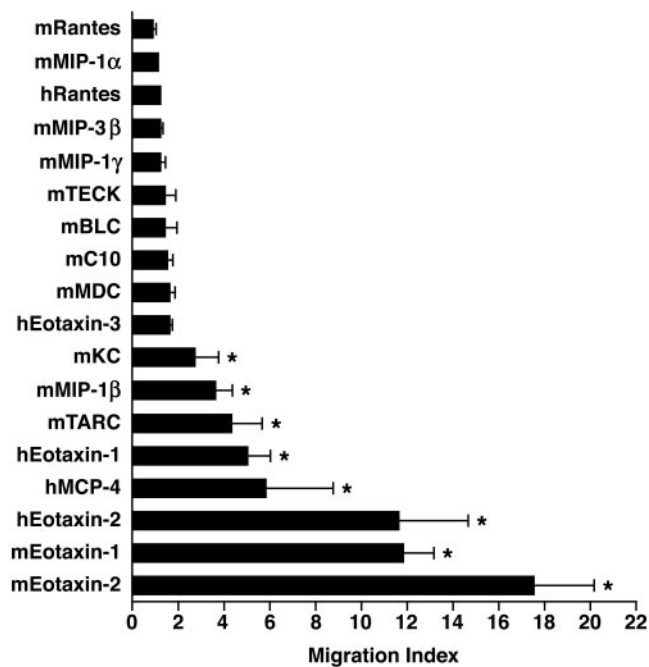


Fig. 4. Mouse eosinophils migrate in response to CCR3 and non-CCR3 ligands. Eosinophil migration in response to various chemokines (100 nM) was investigated using a Transwell insert assay system. Data are expressed as a migration index, as described in Materials and Methods. All chemokines were purchased from R&D Systems with the exception of m eotaxin-1 and m eotaxin-2 (Peprotech). Values presented are means \pm SE of duplicate determinations conducted on three separate occasions. *, Significantly different ($P < 0.05$) from baseline.

jected to a series of alignment parameters in order to identify specific evolutionary relationships among these chemoattractive cytokines (**Fig. 6A**). The amino acid sequences of these proteins as well as the nucleotide sequences of the respective genes were used in jackknife resampling analyses to generate a molecular cladogram describing the evolutionary relationships among these chemokines (**Fig. 6B**). These analyses yield relatively stable relationships among the 18 genes/proteins assessed and show that nearly all chemokines with substantive agonist activities on mouse eosinophils belong to a single clade with high jackknife support. The most parsimonious mapping of agonist activities in the cladogram of **Figure 6B** was to assume that the activities arose independently in all lineages

where activity occurs and to assume five independent evolutionary gains of agonist activity.

DISCUSSION

Three human eotaxin genes (eotaxin-1 [40], eotaxin-2 [41], and eotaxin-3 [24]) and two known mouse eotaxin genes (eotaxin-1 [32] and eotaxin-2 [33]) have been identified and characterized as CCR3 ligands. Surprisingly, the sequence identity among these eosinophil-selective chemokines is only 34–39% within either species, and although human and mouse eotaxin-1 and -2 are likely orthologous pairs (each pair displaying $\sim 60\%$ identity), a mouse orthologue of human eotaxin-3 has yet to be identified. Although CCR3 has also been identified on human mast cells, basophils, and T cells, the only mouse leukocyte shown to express CCR3 is the eosinophil [42]. In vitro migration assays using purified mouse eosinophils demonstrated that mouse eotaxin-1 reached half-maximal responses at similar doses to eotaxin-2; however, the maximal responses to mouse eotaxin-2 were higher relative to eotaxin-1. The increased response of mouse eosinophils to eotaxin-2 compared with eotaxin-1 parallels previous studies of human eosinophils noting a similar pattern of chemokine responsiveness [41], but is in contrast to a recent study characterizing mouse eotaxins using mixed splenocytes from IL-5 transgenic mice, which demonstrates eotaxin-1 to be more potent than eotaxin-2 [33]. These differences are likely attributable to the cell populations studied. The data presented here and the studies on human eotaxins used eosinophils that were purified to $>98\%$, whereas the conflicting study [33] used a population of cells that were only 30–40% eosinophils. Human MCP-4 was also a potent chemokine, exhibiting significant activity toward mouse eosinophils and consistent with its demonstrated activity for human eosinophils [27]. This is not unexpected given that MCP-4 shares high sequence identity with mouse and human eotaxin-1 and binds CCR3. Human eotaxin-3 is the only chemokine of this group not to exert effects on mouse eosinophil migration. However, because human eotaxin-3 has only been reported to induce human eosinophil migration at a high dose (1 μM) [24], the lack of effects at the nanomolar concentrations used in this study is not surprising.

The data presented here demonstrate that although MIP-1 α can bind to purified mouse eosinophils, this chemokine is

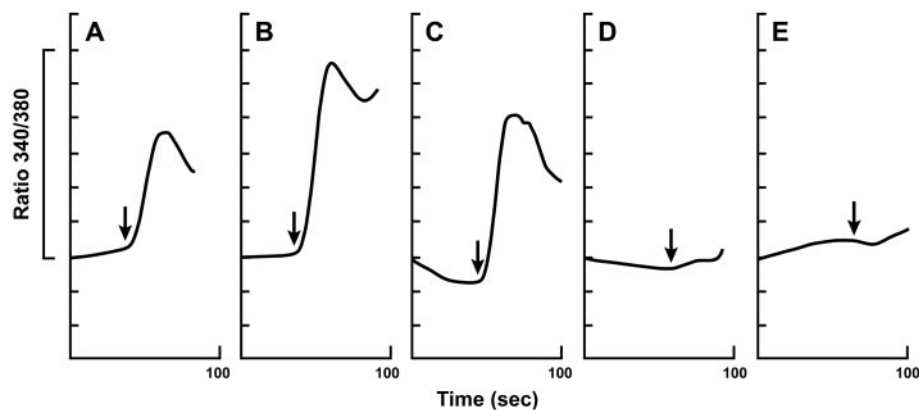


Fig. 5. TARC induces intracellular calcium flux in purified eosinophils. Calcium flux was assessed in eosinophils exposed to chemokines at concentrations that induced in vitro migration: (A) m eotaxin-1 (30 nM), (B) m eotaxin-2 (30 nM), (C) m TARC (100 nM), (D) m MIP-1 β (100 nM), and (E) m KC (100 nM). Arrows indicate addition of chemokine. Data are presented as ratio of fluorescence at 340 and 380 nm. Scale bar equals a 340/380 fluorescence ratio of 1. Data are representative of values obtained from independent experiments conducted on at least three separate occasions.

A

hEotaxin-1 MKVS--ALLWLLLIAAAFSPQGLAGP--A-----SVP-TTC-CFNLANKRIPL---
hEotaxin-2 MAGL--MTIVTSLFLVGLVCAHIIPTG--SV-----VIP-SPC-CMFFVSKRIPE---
hEotaxin-3 MMG--LSLASAVLLASLLSLHLGTATRGS-----DIS-KTC-CFYSHKPLPW---
hMCP MKVS--AVLLCLLLMTAAFPQGLAQPDAL-----NVP-STC-CFTFSSKKISL---
hRANTES MKVS--AARLAVILITATLCA PASAPASPYSS-----DT--TPC-CFAYIARPLPR---
mBLC MRL---STATL LLLASCLSPHGILEAH-----YTN-LKCRSGVISTVVLG---
mMIP1β MKLC--VSALSLLLVAAPCAPGFSAPMGS-----DPP-TSC-CFYSYTSRQLHR---
mC10 MRNS--KTAISFF ILVAVLGSAQGLIQEME--KE-----DRRYNPP I IHQGFQDTS--SDC-CFSYAT-QIPC---
mMIP3β MAPR--VTPLLAFSLVLWTFPAPTGGAN-----DA--EDC-CLSVTRQPIPG---
mEotaxin-1 MQSS--TALLFLLLTVTSFTSQVLAHP--G-----SIP-TSC-CFIMTSKKIPN---
mEotaxin-2 MAGS--ATIVAGLLLVACACCIFFID--SV-----TIP-SSC-CTSFSISKIPE---
mMDC MATLRVPLLVALVLLAVAIQTS DAGPYGA-----NVEDSIC-CQDYIRHPLPS---
mKC MIPATRSLLCAALLLLATSRLATGAPIAN-----E--LRCQCLQTM A-GIHL---
mRANTES MKIS--AAALTI ILTAAALCTPAPASPYGS-----DT--TPC-CFAYLSLELPR---
mTARC MRSL--QMLLAALLLGTFLQHARAAR--AT-----NVG-REC-CLDYFKGAIP I---
mTECK MKLW--LFAVLVACFVGAWMPVVAHQG--AF-----EDC-CLGYQH-RIKWNVL---
mMIPγ MKPF--HTALSFLILTTALGIWAQITHTATETKEVQSSLKAQQGLEIEMPHMGFQD--S-SDC-CLSYNS-RIQC---
mMIPα MKVSTTALAVLLCTMTLCNQVFSAPYGAD-----TP-TAC-CFSYSR-KIPR---

hEotaxin-1 QRLESYRRITS--GKCPQKAVIFKTKLAKDICADPKKK-----W-----VQD-SMKYLDQKS abcdefg
hEotaxin-2 NRVVS YQLSSR--STCLKGGVIFTTKKGQFCGDPKQE-----W-----VQR-YMKNLDAKQ 1211001
hEotaxin-3 TWVRSY-EFTS--NSCSQRAVIFTTRGRKKVCTHPRKK-----W-----VQK-YISLLKTPK 2011011
hMCP QRLKSY-VITT--SRCPQKAVIFRTRKLGKEICADPKKE-----W-----VQN-YMKHLGRKA 1011011
hRANTES AHKEY--FYTS--GKCSNPAVVFVTRKNRQVCANPEKK-----W-----VRE-YINSLEMS- 1001011
mBLC NIIDRIQVTPPPGNGCPKTEVVWTKMKKVICVNPRAK-----W-----LQR-LLRHVQSKS 3010000
mMIP1β SFVMDY-YETS--SLCSKPAVFLTKRGRQICANPSEP-----W-----VTE-YMSDLELN- 1011011
mC10 KRPIYY-FPTS--GGC IKPGIIFISRRGTQVADPSSDR-----R-----VQR-CLSTLKGQ- 1011111
mMIP3β NIVKAFRYLLNEDGCRVPAVVFVTLRGLQCAPPDQP-----W-----VDR-IIRRLKSS 1001000
mEotaxin-1 TLLKSYKRITN--NRCTLKAIVFKTRLKGKEICADPKKK-----W-----VQD-ATKHLDQKL 1211001
mEotaxin-2 NRVVS YQLANG--SICPKAGVIFITKKGKICIDPKLL-----W-----VQR-HIQKLDKAK 1111001
mMDC RLKVEF-FWTS--KSCRKPGVVLITVKNRDI CADPQV-----W-----VKK-LLHLKLS-- 0071011
mKC KNIQSLKVLPSGPHCTQTEVIATLKNGREACLDPEAP-----L-----VQK-IVQKMLKGV 0050100
mRANTES AHVKEY--FYTS--SKCSNLA VVFVTRNRNQCANPEKK-----W-----VQE-YINYLEMS- 1001011
mTARC RKLVSW-YKTS--VECSRDAIVFLTVQGLKICADPKDK-----H-----VKK-AIRLVKNPR 1111011
mTECK RHARNYHQEVS GSNLRAVRFYFRQ--KVVCGNPEDMNVKRAIRLLTARKRLVHWKSADSDS QTERKKS NHMKS KV 1121100
mMIPγ SRF IGY-FPTS--GGCTRP G IIFISKRGPQVCANPSSDR-----R-----VQR-CIERLEKNS 1031111
mMIPα -QF IVDYFETSS--LCSQPGVIFLTKRNRQICADSKET-----W-----VQE-YITDLELNA 0041100

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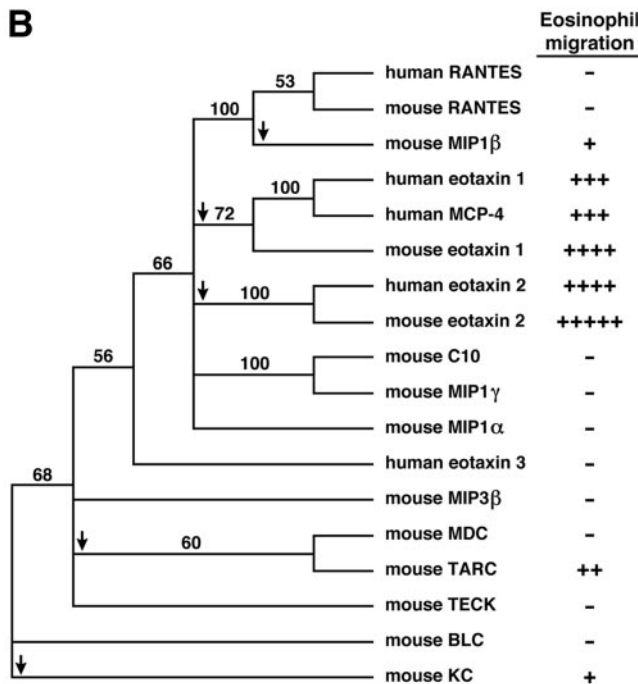


Fig. 6. Amino acid sequence alignment and evolutionary relationships between mouse and human chemokines. (A) Amino acid alignment of the chemokines examined for eosinophil activity. The letters above the alignment (a–g) indicate positions that were recoded as described in Materials and Methods. (B) Molecular cladogram derived from analyses using parsimony for matrices generated with a gap penalty of 5.0. The cladogram is a strict consensus product of two parsimony trees. The numbers on the nodes indicate the percentage of replicates, where the node is retained in the jackknife resampling. Arrows indicate nodes where chemokine agonist activity is mapped in the most parsimonious scenario. Nucleotide and amino acid sequences were obtained from Genbank accession numbers as follows: mouse eotaxin-2 (AF244367), human eotaxin-2 (U85768), mouse eotaxin-1 (U40672), human eotaxin-1 (D49372), human MCP-4 (U46767), mouse TARC (AJ242587), mouse MIP-1β (M35590), human eotaxin-3 (AB010447), mouse KC (J04596), mouse MDC (AF052505), mouse C10 (M58004), mouse BLC (AF044196), mouse TECK (U86358), mouse MIP-1γ (U49513), mouse MIP-3β (AF059208), human RANTES (M21121), mouse RANTES (M77747), and mouse MIP-1α (X12531).

unable to elicit migration of these cells. This apparent paradox is reflective of the literature that abounds with conflicting data concerning the ability of MIP-1α to induce human eosinophil migration [18, 29, 43–46] or eosinophil recruitment in mouse models [6, 31, 47, 48]. In the case of human eosinophils, the study by Sabroe et al. [45], examining eosinophils from multiple donors, provides an explanation to resolve this issue by demonstrating that MIP-1α responses are only observed in ~20% of individuals. The studies in mice have been particularly problematic because these in vitro studies have been

performed on eosinophils whose purity varied from 30% to 99%, and so possible indirect effects of MIP-1α on other cell types may have influenced eosinophil migration. This hypothesis is supported by the present study in which MIP-1α has no effects on purified eosinophils, but exerts some effect on eosinophils when they are studied as a mixed population of leukocytes. The sensitivity of this effect is apparently significant, because even in a mouse eosinophil assay system, comparable with the one used here, Post and colleagues [31] demonstrated that MIP-1α elicits a small (approximately two-

to threefold) increase in eosinophil migration. The reason for this discrepancy is unclear, but may be associated with the use of an eosinophil population of lower purity (85–92%) or a subtle phenotypic difference in eosinophils isolated from transgenic mice with different circulating IL-5 levels. This issue may also complicate the interpretation of *in vivo* studies that support MIP-1 α as an important chemokine in eosinophil recruitment as indirect effects of MIP-1 α , as the mechanism influencing tissue eosinophil accumulation, cannot be eliminated [6, 47, 48].

RANTES is a potent human eosinophil chemoattractant [49] that can bind to CCR1 [50] and CCR3 [18, 19], but appears to exert its effects on eosinophils primarily through CCR3 [51]. However, RANTES displayed no chemotactic activity on mouse eosinophils using two different assay systems. This conclusion is supported by *in vivo* studies demonstrating that intradermal injection of eotaxin, but not RANTES, in the mouse skin recruits eosinophils selectively [47], and transgenic overexpression of RANTES in the mouse lung recruits neutrophils selectively, but not eosinophils [52]. It is interesting that mouse RANTES is unable to displace ¹²⁵I-eotaxin binding on mouse CCR3 transfected cells (B.L.D., unpublished results), suggesting that the loss of chemotaxis induced by mouse RANTES is a consequence of the inability to bind mouse CCR3. The reason for this disparity is unknown, but likely reflects evolutionarily significant selective pressures in one or both species.

The ability of mouse KC, MIP-1 β , and TARC to induce eosinophil migration in these assays suggests that additional chemokine receptors may represent unique pathways for eosinophil migration and function. The biology of KC, in particular, has been well characterized, and it is known to bind through the chemokine receptor CXCR2 [53]. The detection of CXCR2 mRNA in the eosinophils used in this study suggests that signaling through this receptor is responsible for the migration induced by KC. It is interesting that human eosinophils are known to express CXCR2 when they are cultured in IL-5 [51], and thus the possibility exists that the eosinophils used in our studies express functional CXCR2 receptors as they were isolated from transgenic mice with elevated, circulating IL-5 levels (400 pg/ml). Nonetheless, because circulating IL-5 levels also increase during allergic inflammation, CXCR2 expression may represent an alternate/additional pathway facilitating eosinophil recruitment *in vivo*.

TARC and MIP-1 β are the least characterized chemokines that demonstrated activity on mouse eosinophils. MIP-1 β has been characterized as a ligand for CCR5 [54], and the detection of mRNA for this receptor in mouse eosinophils may explain the activity of this chemokine in these studies. The receptor for TARC mediating the migration of eosinophils remains problematic as there is conflicting evidence for TARC as a CCR8 ligand [55–57] but strong evidence for TARC as a CCR4 ligand [58]. Garlisi et al. [55] reported that TARC bound to CCR8 receptors in transfected cells only at very high (i.e., nonphysiologically relevant) concentrations (>250 nM) to compete with the CCR8 ligand I-309. However, in natural killer (NK) cells, TARC was able to compete away I-309 with an IC₅₀ of 1.6 nM [57]. This may reflect a difference in transfected versus primary cells or a difference in cell type

receptor usage (T cells vs. NK cells vs. eosinophils). In either case, no previous evidence exists indicating that these chemokines are eosinophil chemoattractants; instead, both chemokines have been implicated in the responses of T cells during allergic, inflammatory responses [59, 60].

It is interesting that m TARC, but not m KC or m MIP-1 β , induced a significant calcium flux in purified eosinophils. An increased intracellular calcium flux is commonly associated with chemokine receptor signaling, but there are many studies that demonstrate chemokine function occurring in conjunction with low or no calcium flux in various cell types including eosinophils [19, 30, 56]. Several reasons could account for this occurrence including calcium-independent signal transduction pathways utilized by various chemokine receptors or receptor expression levels being too low to evoke a measurable calcium flux within the sensitivity of the assay. In the case of CCR3, a recent study indicates that chemokine binding, intracellular calcium flux, and migration responses are not coupled tightly [43]. Furthermore, given that all of the eotaxin chemokines studied to date bind to eosinophils with similar affinities ($K_d \sim 1\text{--}5$ nM) [18, 23, 24] but exhibit different efficacies and potencies in terms of migration, raises the question of how a chemokine receptor distinguishes between various ligands and modulates the intracellular signaling pathways necessary to achieve graded responses.

An important point to consider in the interpretation of these studies is the possibility that IL-5 has uncoupled or desensitized some of the receptor binding or signaling responses, which occur normally in eosinophils. This would explain why, even with strong binding of MIP-1 α to the eosinophils, the binding fails to result in migration. Furthermore, the expression of chemokine receptors such as CCR2 and CCR5 may represent “decoy” receptors for ligands like RANTES and MIP-1 α , which can bind chemokine but do not elicit intracellular signaling or eosinophil migration. This has been demonstrated previously on dendritic cells and monocytes exposed continuously to the cytokine IL-10 [61].

The tertiary structural homology between chemokines ultimately predicts their binding and signaling properties. However, primary sequence similarities also provide useful data for the determination of relationships between proteins. The ability of evolutionarily diverse chemokines outlying the large eosinophil chemotactic clade (mouse TARC and mouse KC) to induce eosinophil migration supports the hypothesis that multiple chemokine signaling pathways have evolved. The commonality of chemokines with eosinophil agonist activities in humans and mice has broad implications and suggests that these chemotactic mechanisms likely predate the evolution of the uniquely eosinophil hematopoietic lineage. For example, CCR3 receptor-ligand interactions represent the dominant signaling pathway leading to eosinophil recruitment in at least two extant mammalian orders (i.e., Rodentia and Primata), suggesting that the advent of CCR3-mediated eosinophil chemotaxis occurred before the major radiation of mammalian orders 75–100 million years ago [62], and thus potentially prior to the evolution of the mammalian eosinophil hematopoietic lineage.

These studies demonstrate that significant differences exist with regard to the control of eosinophil migration by chemokines between humans and mice and suggest that additional

chemokine/receptor interactions may regulate eosinophil migration. These differences stress the importance of increased characterization of the mouse as a model of human diseases. The greater understanding of the mouse as a model system will no doubt provide invaluable insights regarding eosinophils and their effector functions in disease.

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REFERENCES

- Gleich, G. J., Adolphson, C. R., Leiferman, K. M. (1993) The biology of the eosinophilic leukocyte. *Annu. Rev. Med.* 44, 85–101.
- Wardlaw, A. J. (1999) Molecular basis for selective eosinophil trafficking in asthma: a multistep paradigm. *J. Allergy Clin. Immunol.* 104, 917–926.
- Kung, T. T., Stelts, D., Zureher, J. A., Watnick, A. S., Jones, H., Mauser, P. J., Fernandez, X., Umland, S., Kreutner, W., Chapman, R. W., Egan, R. W. (1994) Mechanisms of allergic pulmonary eosinophilia in the mouse. *J. Allergy Clin. Immunol.* 94, 1217–1224.
- Nakajima, H., Iwamoto, I., Tomoe, S., Matsumura, R., Tomioka, H., Takatsu, K., Yoshida, S. (1992) CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am. Rev. Respir. Dis.* 146, 374–377.
- Renz, H., Smith, H. R., Henson, J. E., Ray, B. S., Irvin, C. G., Gelfand, E. W. (1992) Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J. Allergy Clin. Immunol.* 89, 1127–1138.
- Gonzalo, J. A., Lloyd, C. M., Wen, D., Albar, J. P., Wells, T. N., Proudfoot, A., Martinez-A, C., Dorf, M., Bjerke, T., Coyle, A. J., Gutierrez-Ramos, J. C. (1998) The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med.* 188, 157–167.
- Bedell, M. A., Jenkins, N. A., Copeland, N. G. (1997) Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. *Genes Dev.* 11, 1–10.
- Sica, A., Wang, J. M., Colotta, F., Dejana, E., Mantovani, A., Oppenheim, J. J., Larsen, C. G., Zachariae, C. O., Matsushima, K. (1990) Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J. Immunol.* 144, 3034–3038.
- Marfaing-Koka, A., Devergne, O., Gorgone, G., Portier, A., Schall, T. J., Galanaud, P., Emilie, D. (1995) Regulation of the production of the RANTES chemokine by endothelial cells. Synergistic induction by IFN-gamma plus TNF-alpha and inhibition by IL-4 and IL-13. *J. Immunol.* 154, 1870–1878.
- Taha, R. A., Minshall, E. M., Miotto, D., Shimbara, A., Luster, A., Hogg, J. C., Hamid, Q. A. (1999) Eotaxin and monocyte chemoattractant protein-4 mRNA expression in small airways of asthmatic and nonasthmatic individuals. *J. Allergy Clin. Immunol.* 103, 476–483.
- Mochizuki, M., Bartels, J., Mallet, A. I., Christophers, E., Schroder, J. M. (1998) IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J. Immunol.* 160, 60–68.
- John, M., Hirst, S. J., Jose, P. J., Robichaud, A., Berkman, N., Witt, C., Twort, C. H., Barnes, P. J., Chung, K. F. (1997) Human airway smooth muscle cells express and release RANTES in response to T helper 1 cytokines: regulation by T helper 2 cytokines and corticosteroids. *J. Immunol.* 158, 1841–1847.
- Pype, J. L., Dupont, L. J., Menten, P., Van Coillie, E., Opendakker, G., Van Damme, J., Chung, K. F., Demedts, M. G., Verleden, G. M. (1999) Expression of monocyte chemoattractant protein (MCP)-1, MCP-2, and MCP-3 by human airway smooth-muscle cells. Modulation by corticosteroids and T-helper 2 cytokines. *Am. J. Respir. Cell Mol. Biol.* 21, 528–536.
- Minshall, E. M., Cameron, L., Lavigne, F., Leung, D. Y., Hamilos, D., Garcia-Zepeda, E. A., Rothenberg, M., Luster, A. D., Hamid, Q. (1997) Eotaxin mRNA and protein expression in chronic sinusitis and allergen-induced nasal responses in seasonal allergic rhinitis. *Am. J. Respir. Cell Mol. Biol.* 17, 683–690.
- Lamkhioued, B., Renzi, P. M., Abi-Younes, S., Garcia-Zepeda, E. A., Allakhverdi, Z., Ghaffar, O., Rothenberg, M. D., Luster, A. D., Hamid, Q. (1997) Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J. Immunol.* 159, 4593–4601.
- Ying, S., Robinson, D. S., Meng, Q., Rottman, J., Kennedy, R., Ringler, D. J., Mackay, C. R., Daugherty, B. L., Springer, M. S., Durham, S. R., Williams, T. J., Kay, A. B. (1997) Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur. J. Immunol.* 27, 3507–3516.
- Baggiolini, M. (1998) Chemokines and leukocyte traffic. *Nature* 392, 565–568.
- Daugherty, B. L., Siciliano, S. J., DeMartino, J. A., Malkowitz, L., Sirotina, A., Springer, M. S. (1996) Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J. Exp. Med.* 183, 2349–2354.
- Ponath, P. D., Qin, S., Post, T. W., Wang, J., Wu, L., Gerard, N. P., Newman, W., Gerard, C., Mackay, C. R. (1996) Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J. Exp. Med.* 183, 2437–2448.
- Schweizer, R. C., Welmers, B. A., Raaijmakers, J. A., Zanen, P., Lambers, J. W., Koenderman, L. (1994) RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5. *Blood* 83, 3697–3704.
- Nagase, H., Miyamasu, M., Yamaguchi, M., Fujisawa, T., Ohta, K., Yamamoto, K., Morita, Y., Hirai, K. (2000) Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation. *J. Immunol.* 164, 5935–5943.
- Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Totty, N. F., Truong, O., Hsuan, J. J., Williams, T. J. (1994) Eotaxin—a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med.* 179, 881–887.
- Forssmann, U., Uguccioni, M., Loetscher, P., Dahinden, C. A., Langen, H., Thelen, M., Baggiolini, M. (1997) Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. *J. Exp. Med.* 185, 2171–2176.
- Kitaura, M., Suzuki, N., Imai, T., Takagi, S., Suzuki, R., Nakajima, T., Hirai, K., Nomiya, H., Yoshie, O. (1999) Molecular cloning of a novel human CC chemokine (eotaxin-3) that is a functional ligand of CC chemokine receptor 3. *J. Biol. Chem.* 274, 27975–27980.
- Weber, M., Uguccioni, M., Ochensberger, B., Baggiolini, M., Clark-Lewis, I., Dahinden, C. A. (1995) Monocyte chemoattractant protein MCP-2 activates human basophil and eosinophil leukocytes similar to MCP-3. *J. Immunol.* 154, 4166–4172.
- Dahinden, C. A., Geiser, T., Brunner, T., von Tschamer, V., Caput, D., Ferrara, P., Minty, A., Baggiolini, M. (1994) Monocyte chemoattractant protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J. Exp. Med.* 179, 751nd756.
- Uguccioni, M., Loetscher, P., Forssmann, U., Dewald, B., Li, H., Lima, S. H., Li, Y., Kreider, B., Garotta, G., Thelen, M., Baggiolini, M. (1996) Monocyte chemoattractant protein 4 (MCP-4), a novel structural and functional analogue of MCP-3 and eotaxin. *J. Exp. Med.* 183, 2379–2384.
- Schall, T. J., Simpson, N. J., Mak, J. Y. (1992) Molecular cloning and expression of the murine RANTES cytokine: structural and functional conservation between mouse and man. *Eur. J. Immunol.* 22, 1477–1481.
- Rot, A., Krieger, M., Brunner, T., Bischoff, S. C., Schall, T. J., Dahinden, C. A. (1992) RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. *J. Exp. Med.* 176, 1489–1495.
- Bochner, B. S., Bickel, C. A., Taylor, M. L., MacGlashan Jr., D. W., Gray, P. W., Raport, C. J., Godiska, R. (1999) Macrophage-derived chemokine induces human eosinophil chemotaxis in a CC chemokine receptor 3- and

- CC chemokine receptor 4-independent manner. *J. Allergy Clin. Immunol.* 103, 527–532.
31. Post, T. W., Bozic, C. R., Rothenberg, M. E., Luster, A. D., Gerard, N., Gerard, C. (1995) Molecular characterization of two murine eosinophil beta chemokine receptors. *J. Immunol.* 155, 5299–5305.
 32. Rothenberg, M. E., Luster, A. D., Leder, P. (1995) Murine eotaxin—an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc. Natl. Acad. Sci. USA* 92, 8960–8964.
 33. Zimmermann, N., Hogan, S. P., Mishra, A., Brandt, E. B., Bodette, T. R., Pope, S. M., Finkelman, F. D., Rothenberg, M. E. (2000) Murine eotaxin-2: a constitutive eosinophil chemokine induced by allergen challenge and IL-4 overexpression. *J. Immunol.* 165, 5839–5846.
 34. Lee, N. A., McGarry, M. P., Larson, K. A., Horton, M. A., Kristensen, A. B., Lee, J. J. (1997) Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *J. Immunol.* 158, 1332–1344.
 35. Fischer, F. R., Santambrogio, L., Luo, Y., Berman, M. A., Hancock, W. W., Dorf, M. E. (2000) Modulation of experimental autoimmune encephalomyelitis: effect of altered peptide ligand on chemokine and chemokine receptor expression. *J. Neuroimmunol.* 110, 195–208.
 36. Thompson, J. D., Higgins, D. G., Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
 37. Agosti, D., Jacobs, D., DeSalle, R. (1995) On combining protein sequences and nucleic acid sequences in phylogenetic analysis: the homeobox protein case. *Cladistics* 11, 211–221.
 38. DeSalle, R., Brower, A. V. Z. (1997) Process partitions, congruence and the independence of characters: inferring relationships among closely related Hawaiian *Drosophila* from multiple gene regions. *Syst. Biol.* 46, 713–719.
 39. Swofford, D. L. (1998) *PAUP* 4.0—Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sunderland, MA, Sinauer Assoc.
 40. Ponath, P. D., Qin, S., Ringler, D. J., Clark-Lewis, I., Wang, J., Kassam, N., Smith, H., Shi, X., Gonzalo, J. A., Newman, W., Gutierrez-Ramos, J. C., Mackay, C. R. (1996) Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J. Clin. Investig.* 97, 604–612.
 41. White, J. R., Imburgia, C., Dul, E., Appelbaum, E., O'Donnell, K., O'Shannessy, D. J., Brawner, M., Fornwald, J., Adamou, J., Elshourbagy, N. A., Kaiser, K., Foley, J. J., Schmidt, D. B., Johanson, K., Macphee, C., Moores, K., McNulty, D., Scott, G. F., Schleimer, R. P., Sarau, H. M. (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.
 42. Grimaldi, J. C., Yu, N. X., Grunig, G., Seymour, B. W., Cottrez, F., Robinson, D. S., Hosken, N., Ferlin, W. G., Wu, X., Soto, H., O'Garra, A., Howard, M. C., Coffman, R. L. (1999) Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). *J. Leukoc. Biol.* 65, 846–853.
 43. Umland, S. P., Wan, Y., Shortall, J., Shah, H., Jakway, J., Garlisi, C. G., Tian, F., Egan, R. W., Billah, M. M. (2000) Receptor reserve analysis of the human CCR3 receptor in eosinophils and CCR3-transfected cells. *J. Leukoc. Biol.* 67, 441–447.
 44. Ebisawa, M., Yamada, T., Bickel, C., Klunk, D., Schleimer, R. P. (1994) Eosinophil transendothelial migration induced by cytokines. III. Effect of the chemokine rantes. *J. Immunol.* 153, 2153–2160.
 45. Sabroe, I., Hartnell, A., Jopling, L. A., Bel, S., Ponath, P. D., Pease, J. E., Collins, P. D., Williams, T. J. (1999) Differential regulation of eosinophil chemokine signaling via CCR3 and non-CCR3 pathways. *J. Immunol.* 162, 2946–2955.
 46. Nagase, H., Yamaguchi, M., Jibiki, S., Yamada, H., Ohta, K., Kawasaki, H., Yoshie, O., Yamamoto, K., Morita, Y., Hirai, K. (1999) Eosinophil chemotaxis by chemokines: a study by a simple photometric assay. *Allergy* 54, 944–950.
 47. Teixeira, M. M., Wells, T. N., Lukacs, N. W., Proudfoot, A. E., Kunkel, S. L., Williams, T. J., Hellewell, P. G. (1997) Chemokine-induced eosinophil recruitment. Evidence of a role for endogenous eotaxin in an in vivo allergy model in mouse skin. *J. Clin. Investig.* 100, 1657–1666.
 48. Campbell, E. M., Kunkel, S. L., Strieter, R. M., Lukacs, N. W. (1998) Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia. *J. Immunol.* 161, 7047–7053.
 49. Kameyoshi, Y., Dorschner, A., Mallet, A. L., Christophers, E., Schroder, J. M. (1992) Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.* 176, 587–592.
 50. Gao, J. L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U., Murphy, P. M. (1993) Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor. *J. Exp. Med.* 177, 1421–1427.
 51. Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., Ponath, P. D., Mackay, C. R. (1997) Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Investig.* 99, 178–184.
 52. Pan, Z. Z., Parkyn, L., Ray, A., Ray, P. (2000) Inducible lung-specific expression of RANTES: preferential recruitment of neutrophils. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 279, L658–L666.
 53. Bozic, C. R., Kolakowski Jr., L. F., Gerard, N. P., Garcia-Rodriguez, C., von Uexkull-Guldenband, C., Conklyn, M. J., Breslow, R., Showell, H. J., Gerard, C. (1995) Expression and biologic characterization of the murine chemokine KC. *J. Immunol.* 154, 6048–6057.
 54. Combadiere, C., Ahuja, S. K., Tiffany, H. L., Murphy, P. M. (1996) Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1(alpha), MIP-1(beta), and RANTES. *J. Leukoc. Biol.* 60, 147–152.
 55. Garlisi, C. G., Xiao, H., Tian, F., Hedrick, J. A., Billah, M. M., Egan, R. W., Umland, S. P. (1999) The assignment of chemokine-chemokine receptor pairs: TARC and MIP-1 beta are not ligands for human CC-chemokine receptor 8. *Eur. J. Immunol.* 29, 3210–3215.
 56. Bernardini, G., Hedrick, J., Sozzani, S., Luini, W., Spinetti, G., Weiss, M., Menon, S., Zlotnik, A., Mantovani, A., Santoni, A., Napolitano, M. (1998) Identification of the CC chemokines TARC and macrophage inflammatory protein-1 beta as novel functional ligands for the CCR8 receptor. *Eur. J. Immunol.* 28, 582–588.
 57. Inngjerdingen, M., Damaj, B., Maghazachi, A. A. (2001) Expression and regulation of chemokine receptors in human natural killer cells. *Blood* 97, 367–375.
 58. Imai, T., Baba, M., Nishimura, M., Kakizaki, M., Takagi, S., Yoshie, O. (1997) The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J. Biol. Chem.* 272, 15036–15042.
 59. Tedla, N., Wang, H. W., McNeil, H. P., Di Girolamo, N., Hampartoumian, T., Wakefield, D., Lloyd, A. (1998) Regulation of T lymphocyte trafficking into lymph nodes during an immune response by the chemokines macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta. *J. Immunol.* 161, 5663–5672.
 60. Kakinuma, T., Nakamura, K., Wakugawa, M., Mitsui, H., Tada, Y., Saeki, H., Torii, H., Asahina, A., Onai, N., Matsushima, K., Tamaki, K. (2001) Thymus and activation-regulated chemokine in atopic dermatitis: serum thymus and activation-regulated chemokine level is closely related with disease activity. *J. Allergy Clin. Immunol.* 107, 535–541.
 61. D'Amico, G., Frascaroli, G., Bianchi, G., Transidico, P., Doni, A., Vecchi, A., Sozzani, S., Allavena, P., Mantovani, A. (2000) Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nat. Immunol.* 1, 387–391.
 62. McKenna, M. G. (1969) The origin and early differentiation of thierian mammals. *Ann. N. Y. Acad. Sci.* 167, 217–240.