Distinct Expression and Function of the Novel Mouse Chemokine Monocyte Chemotactic Protein-5 in Lung Allergic Inflammation

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Summary

We have cloned a novel mouse CC chemokine cDNA from the lung during an allergic inflammatory reaction. The protein encoded by this cDNA is chemotactic for eosinophils, monocytes, and lymphocytes in vitro and in vivo. Based on its similarities in sequence and function with other CC chemokines, we have named it mouse monocyte chemotactic protein-5 (mMCP-5). Under noninflammatory conditions, expression of mMCP-5 in the lymph nodes and thymus is constitutive and is generally restricted to stromal cells. Neutralization of mMCP-5 protein with specific antibodies during an allergic inflammatory reaction in vivo resulted in a reduction in the number of eosinophils that accumulated in the lung. Moreover, mMCP-5 mRNA expression in vivo is regulated differently from that of other major CC chemokines in the lung during the allergic reaction, including Eotaxin. The presence of lymphocytes is essential for expression of mMCP-5 by alveolar macrophages and smooth muscle cells in the lung, and the induction of mMCP-5 RNA occurs earlier than that of the eosinophil chemokine Eotaxin during allergic inflammation. In contrast to Eotaxin, mRNA for mMCP-5 can be produced by mast cells. From these results, we postulate that mMCP-5 plays a pivotal role during the early stages of allergic lung inflammation.

The late phase of an asthmatic reaction is mediated by a swarm of leukocytes that are recruited to the lung by cytokines and chemotactic factors (1). The mechanism(s) by which the different populations of leukocytes are recruited and how they regulate the attraction and function of other leukocytes present at the inflammatory site is poorly understood. However, there is evidence to suggest that the accumulation of eosinophils at sites of allergic reactions may be directly associated with the presence of lymphocytes and with the production by T cells of cytokines, which are known to stimulate eosinophil maturation, activation, and survival, or to modulate the expression of adhesion molecules (2, 3).

At the present time, the accepted paradigm for leukocyte extravasation is that the selective homing of particular circulating leukocytes into inflamed tissues takes place via the generation of chemotactic gradients created by chemoattractants or by specific cytokines termed chemokines (4, 5). Most chemokines can be grouped as members of either the CXC (α) or CC (β) family to denote the spacing of the first two cysteine residues of the mature proteins. The CXC

chemokine family seems to act preferentially on neutrophils and includes, among others, platelet factor 4, NAP-1/IL-8, gro, IP-10, and ENA-78. The CC family has been described as attracting several leukocyte subsets, but not neutrophils, and includes macrophage inflammatory protein (MIP)[1]-1α, -β, monocyte chemotactic protein-1 (MCP-1)/JE, MCP-2, MCP-3, Eotaxin, and RANTES (6). The relevance of chemokines in the migration of leukocytes to inflammatory sites in vivo has been demonstrated in various experimental systems of differing complexity (7–9).

Because of their broad chemotactic specificities, CC chemokines could play a central role in the development and maintenance of the leukocytic infiltration found in the lung during allergic inflammation (eosinophils, lymphocytes, monocytes, etc., and lack of a significant and/or predominant infiltration of neutrophils). We and others have

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[1] Abbreviations used in this paper: BMCMC, bone marrow–cultured mast cells; h, human; m, mouse; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RT, reverse transcriptase.

identified and studied several CC chemokines, including mouse Eotaxin, in the lungs of mice that were rendered eosinophilic by repeated exposure to aerosolized OVA (10–13).

The work reported here represents our continued efforts in the identification and study of CC chemokines that could be integral in promoting the complex leukocyte infiltration that is present in the lung during allergic reactions. We have isolated a novel cDNA from mouse hypereosinophilic lungs by PCR amplification based on consensus sequences that are present in CC chemokine genes. This novel mouse CC chemokine has strong homology with human (h)MCP-1. The chemotactic activity of the protein encoded by this gene in vitro, as well as after in vivo administration, and the regulation of the expression and function of this gene during lung eosinophilia are presented here.

Materials and Methods

Mice and In Vivo Procedures. 8–10-wk-old male and female C57BL/6J and RAG-1–deficient mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and kept in the Center for Blood Research Specific Pathogen Free mouse facility (Harvard Medical School). CD3ε-transgenic mice (14) were kindly provided by Dr. C. Terhorst (Beth Israel Hospital, Boston, MA). OVA-induced pulmonary eosinophilia (Sigma Immunochemicals, St. Louis, MO) was generated in these mice (three individual experiments, $n = 10$) as previously described (10).

Peritoneal recruitment assays in vivo with mouse (m)MCP-5 protein were performed after injection of 400 or 800 µl i.p. of mMCP-5 recombinant protein–containing conditioned media (see below) or control conditioned media. At different time points after injection (0, 1, 2, 3, or 4 h), (three individual experiments, $n = 8$–13 for test and control mice per time point and dose), leukocytes recovered from peritoneal lavage were analyzed and enumerated. For the coinjection experiments, bacterial rmEotaxin (0.5 µg/200 µl per mouse; Peprotech, Inc., Rocky Hill, NJ) was administered intraperitoneally simultaneously with either 400 or 800 µl of mMCP-5 containing conditioned media. In one series of experiments, lower doses of Eotaxin (0.2 µg/mouse) were coinjected with lower doses of mMCP-5 (100 µl/mouse). Control mice were injected intraperitoneally with (a) rmEotaxin protein and control conditioned media; (b) PBS and mMCP-5–containing conditioned media; and (c) PBS and control conditioned media. All doses and volumes were exactly the same as those given to test mice. At different time points after coinjection of mMCP-5–containing conditioned media and rmEotaxin (1, 2, 3, or 4 h; three individual experiments, $n = 4$–12, for test and control mice per time point and dose), recovered cells from the peritoneum were analyzed. In one series of blocking experiments, mice were injected with affinity-purified neutralizing polyclonal antibodies against mMCP-5 (20 µg/mouse, i.v.) 30 min before OVA administration on days 20 and 21, and then analyzed 3 h after allergen challenge on day 21. OVA-treated control mice were injected with the same amount of control Ab (purified rabbit Ig fraction; Dako Corp., Carpinteria, CA) at the time points indicated during treatment.

Cloning of cDNA by Reverse Transcription (RT)-PCR. A partial cDNA fragment of the mMCP-5 gene was cloned from RNA extracted from eosinophilic lungs of OVA-sensitized mice (10). To clone the full-length mouse mMCP-5 cDNA, we followed the same two-step PCR amplification strategy that we used recently to clone mouse Eotaxin (10). Specific primers used for every step during the cloning were as follows: first step round 1, 5' primer 1 (a chimeric primer containing (dT)$_{17}$ and sequence of the T$_7$ promoter: 5'-TAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT) and 3' primer (5'-CTCCTTATCCAGTATGGTCC); first step round 2, 5' primer 2 (sequence of the T$_7$ promoter: 5'-TAATACGACTCACTATAGGG) and 3' primer (designed nested to first 3'-primer from the sequence of the cloned fragment: 5'-ACAGCTTCCCGGGGACACTG); second step round 1, primers 1 and gene specific 5' primer (designed from the 5' sequence of the cloned fragment: 5'-AGAGACACTGGTTCCTGAC); second step round 2, primers 2 and a second gene specific primer (a primer containing sequences nested to first 5' primer: 5'-TCTCCCTCCACCATGCAGAG).

Production of Recombinant mMCP-5 Protein in p3X63 Myeloma Cells. The entire coding sequence (from 30 to 450 in the nucleotide sequence) of mMCP-5 cDNA was cloned into the expression vector pEFpuro in the sense or antisense orientation and transfected into p3X63 myeloma cells as before (10). To partially purify mMCP-5 from the tissue culture conditioned media (15), 20 ml of mMCP-5–containing or control conditioned media was applied to a 1-ml Heparin column (Pharmacia Fine Chemicals, Piscataway, NJ), as recommended by supplier. The eluate was applied to a cation exchange column (mono S; Pharmacia) and eluted with a linear gradient from 0 to 1 M NaCl in 50 mM acetate buffer, pH 5.0. This eluate was diluted 2.5 times and then used for the assays shown in Fig. 2 *A* (indicated as H.C.). Endotoxin levels were estimated less than 0.1 ng/µg (or 75 U) of MCP-5, as assessed by the LAL assay (BioWhittaker, Inc., Walkersville, MD).

In Vitro Chemotaxis. The in vitro migration of leukocytes to recombinant mMCP-5 was evaluated, in duplicate, using a 24-well transwell chamber with a 5-µm pore (Costar, Cambridge, MA), as described previously (10). For neutralization experiments, 1 or 10 µg/ml of affinity-purified anti–mMCP-5 polyclonal antibodies were preincubated with mMCP-5–containing or control conditioned media for 15 min at 37°C and then used for eosinophil chemotaxis as described above. Purified rabbit Ig fraction (Dako) was used as a control in this assay. After incubation, the transwells were removed and the number of cells per well counted in the FACScan® (Becton Dickinson & Co., Mountain View, CA) by passing each sample for a constant predetermined time period. A constant gate was assigned for each leukocyte subpopulation in the SSC/FSC window and was used for every sample. When necessary, samples were also stained for cell lineage–specific markers, and were analyzed as explained below. The chemotactic index was calculated as the number of cells that migrated to the sample divided by the number of cells that migrated to the negative control (control conditioned media).

Eosinophils were purified from the peritoneum of IL-5–transgenic mice as described previously (10). Monocytes/macrophages and neutrophils were obtained from the bone marrow or peritoneum after injection of thioglycollate and percoll gradient separation (15). Lymphocytes were obtained from the peripheral blood, spleen, LN, or bone marrow after erythrocyte lysis and immunomagnetic depletion of Mac-1$^+$ and Gr-1$^+$ cells, as explained above.

Flow cytometry was performed to determine the cell lineage of migratory cells in selected experiments. The migratory cells from the assay were incubated with anti-Fc receptor mAb (2.492; PharMingen), and were then stained with each one of the following antibodies individually: Gr-1 (8C5), Mac-1 (CD11b), B220 (CD45R),

IgM, or Thy1.2, conjugated with FITC or PE (PharMingen, San Diego, CA). Dead cells were excluded by propidium iodide (Sigma) incorporation. Flow cytometry data were acquired with a FACScan® cytometer, and were analyzed with CELLQUEST software.

Generation and Screening of Polyclonal Sera and mAbs Anti–mMCP-5. Polyclonal sera against mMCP-5 was generated according to standard methods using the synthetic peptide CADP-KEKWVKNSINHLDKTS, covering amino acids 52–71 in the mMCP-5 peptide sequence, coupled to KLH (Pierce Chemical Co., Rockford, IL; 17). Rabbit serum was first depleted of anti–mouse IgG antibodies by passage over a mouse IgG column, and anti–mMCP-5 antibodies were purified from the flowthrough on a mMCP-5 peptide (52–71) affinity column. Antibody titers were determined by ELISA.

10-wk-old Wistar rats were immunized in the hind footpad with the KLH-coupled mMCP-5 peptide, and cell fusions were performed as described before (18). Positive supernatants were studied in Western blot analysis against mMCP-5 that was produced by transfected cells. A total of nine mAbs were obtained and characterized by Western blotting and immunostaining. Four mAbs recognized mMCP-5 epitopes that were present in frozen sections. One of those, ZY2A11, was used for the experiments described here.

Affinity-purified rabbit polyclonal antibodies and rat mAbs were shown to recognize a specific band in a Western blot against mMCP-5–containing conditioned media, but not against the conditioned media of mock-transfected cells (data not shown). This band was specifically competed by preincubation the Ab preparations with the mMCP-5 (52–71) peptide used for immunization (data not shown).

Southern and Northern Blots. Both were performed following standard methods and the following probes: mMCP-5 (0.29-kb fragment cloned from 5' RACE), Eotaxin (10), RANTES (19), and MIP-1α (20).

Measurement of mMCP-5 and mEotaxin mRNA Expression by RT-PCR. For RT-PCR, we followed the methods described before (10). The specific primers for mMCP-5 used in PCR are as following: 5' primer, 5'-TCTCCCTCCACCATGCAGAG and 3' primer, 5'-CTCCTTATCCAGTATGGTCC.

The macrophage cell lines RAW287 and P388D1 were cultured with TNF-α (20 ng/ml) for 24 h, and the endothelial cell line bEnd3 and the fibroblast cell line NIH3T3 were cultured with PMA/Ionomycin (10:500 ng/ml) for 8 h. Macrophages were freshly isolated from mouse peritoneal cavity 48 h after injection of thioglycollate (1 ml/mouse), and were cultured in vitro with TNF-α (20 ng/ml) or IFN-γ respectively for 24 h.

The mast cells studied include a cloned, growth factor–independent mast cell line (Cl.MC/C57.1 [21]) or primary cultures of bone marrow–cultured mast cells (BMCMC). Primary cultures of BMCMC were generated from bone marrow cells isolated from BALB/c mice as described previously (21). BMCMC were used for experiments at 4–6 wk, at which time mast cells represented >99% of the cells, as determined by neutral red staining.

Immunohistochemical Phenotyping and Quantitation of Leukocytes. The number and type of leukocytes migrating in response to mMCP-5 recombinant protein or to both chemokines (rmMCP-5 protein and rmEotaxin protein) in peritoneal lavage fluid was determined as described before (10). To determine the number of lymphocytes, monocytes, and macrophages, slides were stained with mAb against Thy 1.2 (53-2.1), Mac-1 (M1/70), CD45R/B220 (RA3-6B2) from PharMingen, and Moma-2 from Biosource International (Camarillo, CA) using an avidin/biotin staining method as described before and counterstained with hematoxylin.

The number of leukocyte subtypes was determined in four high power fields (magnification of 400; total area = 0.5 mm²) per slide (duplicate slides per mouse and time point were examined). Monocytes and macrophages were distinguished from lymphocytes on the basis of expression of Mac-1 and Moma-2 on their surface and from each other, based on the difference in size and granularity observed after Giemsa counterstaining by light microscopy or by flow cytometry.

Immunohistochemical Staining of Lungs and Lymphoid Tissue for mMCP-5. Sections of lungs from OVA-treated mice and of inguinal LN and thymi from unchallenged mice were fixed and stained with an mAb directed against mMCP-5 using an avidin/biotin staining method. Sections were overlaid with 20% normal rabbit serum in PBS for 15 min. Lungs were incubated with neat anti–mMCP-5 (ZY2A11) culture supernatant, and LN and thymi were incubated with anti–mMCP-5 ascites at 1/100, both overnight at 4°C. Endogenous peroxide was subsequently blocked. Bound mAb was visualized by incubation with biotinylated rabbit anti–rat Ig diluted in 10% normal mouse serum PBS, and then with streptavidin peroxidase complex prepared according to manufacturer's instructions (all from Dako), and incubated for 1 h. Finally, the slides were flooded with peroxidase substrate solution before counterstaining with hematoxylin. Control sections were included where mAb, biotinylated anti–rat Ig, or streptavidin complex were selectively omitted. Control slides of lung were also stained with an isotype-matched negative control antibody instead of primary antibody.

Results

Cloning, Mapping, and Structural Analysis of mMCP-5. We have used degenerated oligonucleotides and PCR to clone novel CC chemokine cDNA sequences from RNAs extracted from eosinophilia lungs (10). Three distinct groups of 150-bp PCR products (including two degenerate primers) with different nucleotide sequences were obtained (10). According to the sequence from one of them, one specific primer was designed to isolate the 5' partial cDNA by 5' RACE cloning strategy. The complete cDNA for this gene was then isolated by 3' RACE cloning, which involved two further rounds of nested amplification by PCR with primers based on the nucleotide sequence of the previously cloned 5' fragment.

The cloned full-length cDNA for this novel gene contains 540 bp, whose nucleotide sequence was confirmed by three independent PCR amplifications. It includes an open reading frame of 341 bases encoding a protein of 104 amino acids, a 5' untranslated region of 55 bp, and a 3' untranslated region of 145 bp (data not shown. GenBank/EMBL/DDBJ accession number U50712). The mature protein is composed of 82 amino acids containing five cysteine residues (Fig. 1 *A*). No potential N-glycosylation sites are present in this protein. The nucleotide similarity of this gene with other CC chemokines is shown in Fig. 1 *B*.

The comparison of the amino acid sequence of this novel gene with those of other members of the CC chemokine family (Fig. 1 *A*) revealed the highest similarity with human MCP-1 and MCP-3 (Fig. 1, *A* and *B*). Because of this and other features described below, this novel mouse CC chemokine gene was named mouse monocyte chemotactic
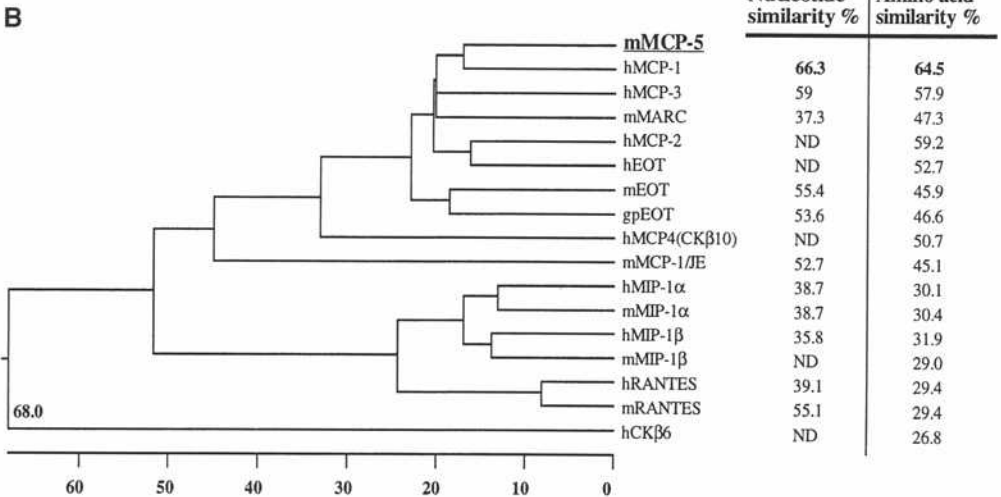
A


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        10            20            30            40
G P D A V S T P V T C C Y N V K Q K I H V R K L K S Y R R I T S S Q C P R E A V I F R T   mMCP-5
Q P D A I N A P V T C C Y N F T N R K I S V Q R L A S Y R R I T S S K C P K E A V I F K T   hMCP1
Q P D S V S I P I T C C F G L V N G K I P F K K L E S Y T R I T N S Q C P Q E A V I F K T   hMCP2
Q P V G I N T S T T C C Y R F I N K K I P K Q R L E S Y R R T T S S H C P R E A V I F K T   hMCP3
Q P D A L N V P S T C C F T F S S K K I S L Q R L K S Y V - I T T S R C P Q K A V I F R T   hMCP-4 (CKβ10)
H P G S I - - P T S C C F I M T S K K I P N T L L K S Y K R I T N N R C T L K A I V F K T   mEot
Q P D A V N A P L T C C Y S F T S K M I P M S R L E S Y K R I T S S R C P K E A V V F V T   mMCP-1/JE

        50            60            70            80
I L D K E I C A D P K E K W V K N S I N H L D K T S Q T F I L E P S C L - - - - G   mMCP-5
I V A K E I C A D P K Q K W V Q D S M D H L D K Q T Q T P K T                       hMCP1
K A D K E V C A D P Q Q K W V Q N S M K L L D Q K S Q T P K - - - - - - - - - - P   hMCP2
K L D K E I C A D P T Q K W V Q D F M K H L D K K T Q T P K L                       hMCP3
K L G K E I C A D P K E K W V Q N Y M K H L G R K A H T L K T                       hMCP-4 (CKβ10)
R L G K E I C A D P K K K W V Q D A T K H L D Q K L Q T P K - - - - - - - - - - P   mEot
K L K R E V C A D P K K E W V Q T Y I K N L D R N Q - - M R S E P T T L F K T A S   mMCP-1/JE
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B

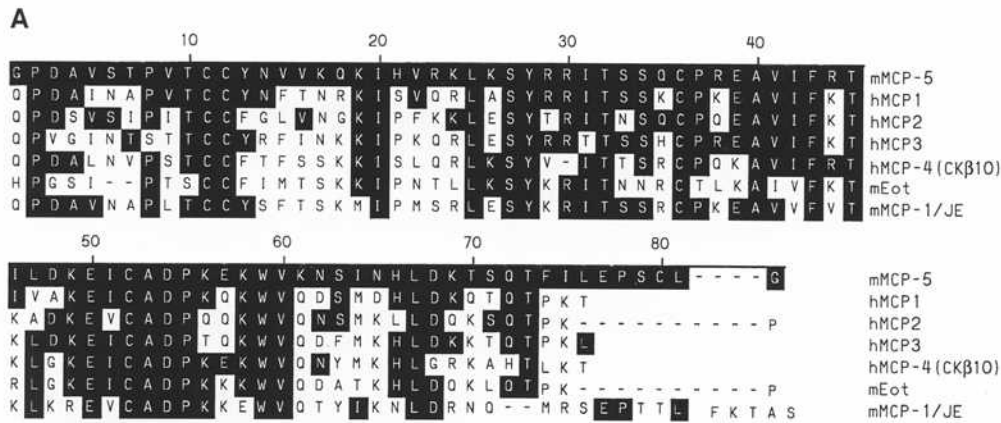|	Nucleotide similarity %	Amino acid similarity %
mMCP-5		
hMCP-1	66.3	64.5
hMCP-3	59	57.9
mMARC	37.3	47.3
hMCP-2	ND	59.2
hEOT	ND	52.7
mEOT	55.4	45.9
gpEOT	53.6	46.6
hMCP4(CKβ10)	ND	50.7
mMCP-1/JE	52.7	45.1
hMIP-1α	38.7	30.1
mMIP-1α	38.7	30.4
hMIP-1β	35.8	31.9
mMIP-1β	ND	29.0
hRANTES	39.1	29.4
mRANTES	55.1	29.4
hCKβ6	ND	26.8

Figure 1. Comparison of the amino acid sequence of the mMCP-5 mature peptide with that of other CC chemokines and the phylogenetic tree of members of the CC chemokine family, including mMCP-5. (*A*) Amino acid sequence alignment of mMCP-5 with other human and mouse CC chemokines. The sequences were aligned using the Clustal method with a PAM250 residue weight table and default settings of gap penalties for pairwise and multiple alignments of 3 and 10, respectively (MegAlign; DNASTAR, Inc., Madison, WI). Amino acid sequences of the mature proteins for the hMCP-1 (35), hMCP-2 (36), hMCP-3 (37), hMCP-4 (38), mMCP-1/JE (39; 1–85 amino acids), and mEot (10) genes were compared to the amino acid sequence of the putative mature mMCP-5 protein. Amino acids identical to mMCP-5 are shaded in black with numbers across the top of the sequence referenced to mMCP-5. The cDNA sequence of mMCP-5 has been deposited in GenBank/EMBL/DDBJ under the accession number U50712. (*B*) Phylogenetic tree of the CC chemokine family including mMCP-5 (bold and underlined). Distances to branch points are proportional to amino acid sequence divergence from predicted ancestral sequences. Percent similarity between the nucleotide sequence of the coding region or the amino acid sequence of the mature peptide of mMCP-5 and those of other CC chemokines are shown at the right of the figure.

protein-5. Comparison of mMCP-5 mature peptide with hMCP-1 mature peptide or with the first 80 amino acids of the mMCP-1/JE mature peptide revealed 65 and 45% similarity, respectively (Fig. 1). mMCP-5 is a basic protein (pI 9.07). The amino acid sequence phylogenetic tree and similarity comparisons with other CC chemokines (Fig. 1 *B*) showed that mMCP-5 is most similar and evolutionarily related to hMCP-1.

The mouse chromosomal location of mMCP-5, which was determined by interspecific back-cross analysis (22), revealed that the single locus encoding for this cDNA is located in the central region of mouse chromosome 11 (data not shown).

To determine the possible existence of a gene homologous to mMCP-5 in humans, a southern blot containing human genomic DNA was hybridized with an mMCP-5 cDNA probe. Under conditions of high stringency, a single hybridizing band was detected (which had a different size from that found when the same blot was hybridized with an hMCP-1 probe; data not shown), suggesting the existence of an mMCP-5 homologue in humans.

In Vitro Chemotactic Responses of Leukocytes to mMCP-5. To characterize the activities of mMCP-5 its expression in mammalian cells was engineered, as we have done previously (reference 10 and Materials and Methods).

Eosinophil chemotaxis to mMCP-5 was examined using mouse eosinophils (90–95% pure) purified from the peritoneal cavity of IL-5–transgenic mice (23), since normal mice do not have appreciable numbers of eosinophils. Conditioned media containing mMCP-5 recombinant protein induced migration of eosinophils in a dose-dependent manner compared to the control conditioned media (Fig. 2 *A*). In three independent experiments with different eosinophil and conditioned media preparations, recombinant mMCP-5
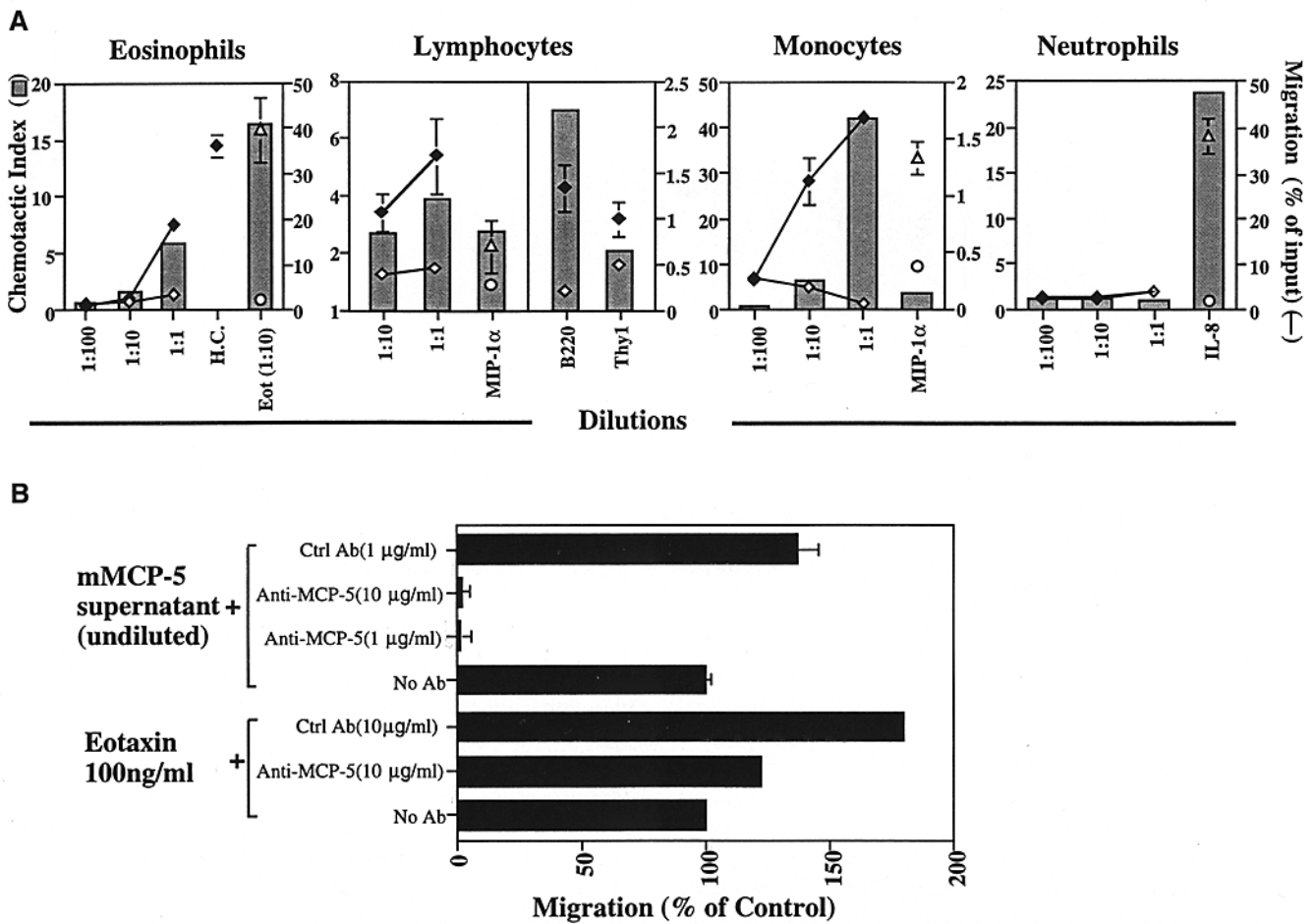
A

B

Figure 2. Chemotactic activity of mMCP-5 on mouse leukocytes in vitro and neutralization of eosinophil chemotaxis to mMCP-5 by anti-mMCP-5 antibodies. (*A*) The indicated mouse leukocyte subtypes were subjected to chemotaxis to different dilutions of the conditioned media containing mMCP-5 (*solid diamonds*) or of the control mock-conditioned media (*empty diamonds*). The bars and symbols show the mean and the range for one representative experiment out of three (or five for lymphocytes and monocytes). Results are expressed as chemotactic index (the ratio between the number of cells that migrated to the sample and the number that migrated to negative control, depicted as bars, axis on left of each plot) and migration (the percentage of the total number of input cells that migrated to the sample, depicted as lines, axis on the right of each plot). Positive controls (*triangles*) used for these experiments were rmEotaxin, rmMIP-1α, and rhIL-8. The migration of leukocytes to assay media are also included (*circle*). In chemotaxis assays of lymphocytes to undiluted mMCP-5–containing conditioned media (*black bars*) or undiluted control conditioned media (*white bars*), migratory cells were stained with anti-B220 or anti-Thy1.2 mAbs, and were analyzed by flow cytometry. (*B*) Eosinophil migration in response to rmMCP-5 is inhibited by specific Abs. The same chemotaxis assays described in *A* for eosinophils were performed. The mMCP-5–containing or control conditioned media were preincubated with or without different concentrations of polyclonal antibodies against mMCP-5 (see Materials and Methods) or with rabbit Ig fraction as a control for 15 min at 37°C and, then used for chemotaxis. The chemotaxis of eosinophils to Eotaxin in the presence of the same Abs was used as control for anti-MCP-5 Ab specificity. The percentage of migration observed in the wells containing control supernatant and/or control Ab was subtracted from their corresponding experimental points. Data are expressed as the percentage of the positive control (chemokine alone), which was considered 100%.

from undiluted conditioned media attracted 10–19% of the input eosinophils in transwell chemotaxis assays. In parallel assays, only 1.1–2.3% of input eosinophils migrated to the control conditioned media (Fig. 2 *A*). Heparin/cation exchange–purified protein (see Materials and Methods) from mMCP-5–containing conditioned media induced the transmigration of ~39% (35–40% migration) of eosinophil input (Fig. 2 *A*).

To evaluate the chemotactic function of mMCP-5 on lymphocytes, four independent experiments were performed and showed that the conditioned media containing mMCP-5 induced the migration of lymphocytes from the bone marrow (Fig. 2 *A*) and from the peripheral blood

(PB) (data not shown and Fig. 2 *A*). We failed to detect reproducible chemotaxis of LN- or spleen-derived lymphocytes to mMCP-5 (data not shown) compared to the control conditioned media. mMCP-5 attracted a subset (~2%) of lymphocytes from both the bone marrow and PB (Fig. 2 *A*). In a separate series of experiments, migratory lymphocytes to mMCP-5 were stained with either anti-B220 or anti-Thy1 mAbs, and the proportion of positive cells for each marker was analyzed by flow cytometry. B lymphocytes displayed a stronger response to mMCP-5 than T lymphocytes, showing a higher chemotactic index (Fig. 2 *A*).

Neutrophils and monocytes/macrophages used for in vitro chemotaxis assays were isolated from either normal
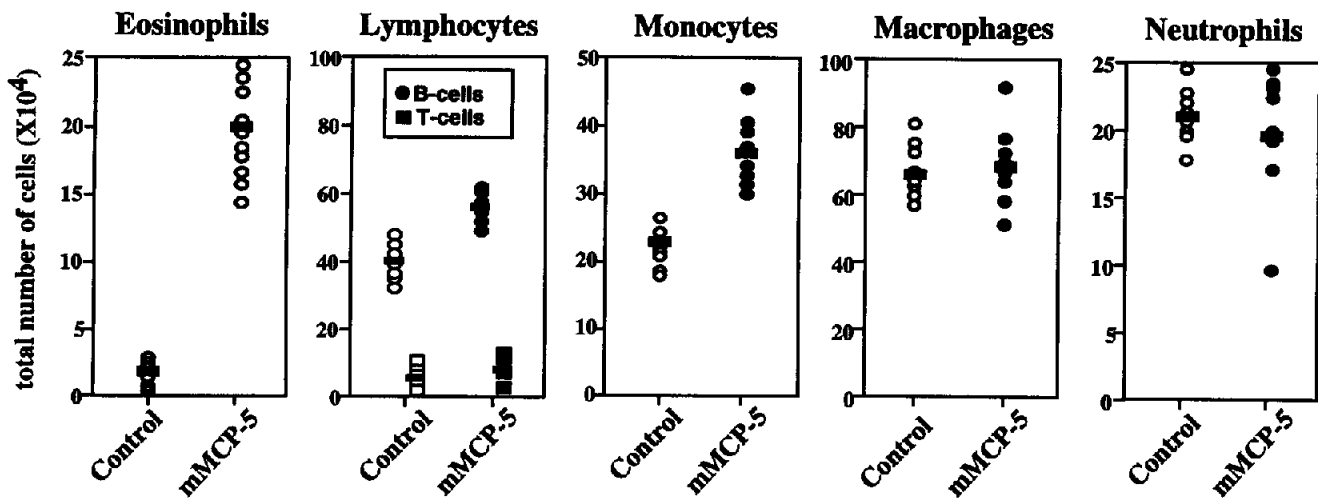
Figure 3. mMCP-5 induced recruitment of leukocytes to the peritoneum. Peritoneal exudate was collected from C57BL/6J mice 2 h after injection of mMCP-5–containing conditioned media (*closed symbols*) or control conditioned media (*open symbols*). Each dot represents one individual mouse analyzed (10 mice per group). The bar in each panel represents the mean of the total number of cells of the leukocyte subtype indicated. For lymphocyte assays, B and T cells were also analyzed separately by immunophenotypic analysis of lymphocytes recruited to the peritoneum after mMCP-5 injection.

mouse bone marrow (neutrophils or monocytes) after erythrocyte lysis and Thy1 + B220 lineage depletion from the peritoneal cavities of IL-5–transgenic mice (monocytes) after Gr-1 + Thy1 + B220 lineage depletion, or from the peritoneum of mice treated previously with thioglycollate (neutrophils or monocytes). Several separate experiments ($n = 9$) with purified populations or enriched cell preparations showed that mMCP-5–containing rather than control conditioned media elicited concentration-dependent chemotaxis of monocytes isolated from BM (data not shown), from the peritoneum of IL-5–transgenic mice (data not shown), or from the peritoneal lavage of mice treated with thioglycollate (Fig. 2 *A*). mMCP-5 supported the chemotactic migration exclusively of microscopically small and nongranular monocytes that clustered in a region characterized by the lowest forward and right angle light scatters found among monocytes (defined as Mac1$^+$ Gr-1$^-$; data not shown) when analyzed by flow cytometry. No significant differences were found between mMCP-5–containing and control conditioned media in the chemotactic migration of neutrophils isolated either from bone marrow (Fig. 2 *A*) or from the peritoneal cavity of thioglycollate-treated mice (not shown). These results were confirmed by the use of mMCP-5 purified by heparin/cation exchange chromatography in these assays, which showed similar chemotactic activity on lymphocytes and monocytes, but not on neutrophils (data not shown).

To attribute unequivocally all the chemotactic activity to eosinophils present in the conditioned media from mMCP-5–transfected cells to mMCP-5, we blocked it in vitro with affinity-purified polyclonal antibodies raised against an mMCP-5 peptide (see Materials and Methods). Anti–mMCP-5 antibodies were able to neutralize almost all the eosinophil chemotactic activity present in the mMCP-5 conditioned media, but did not affect the chemotaxis of eosinophils to Eotaxin (Fig. 2 *B*). Similarly, anti–MCP-5

antibodies were able to neutralize the migration of lymphocytes and monocytes to mMCP-5 in vitro or in vivo (data not shown).

In Vivo Peritoneal Recruitment of Leukocytes to mMCP-5. The injection of mMCP-5–containing conditioned media in mice resulted in a moderate increase in the total number of peritoneal cells that maximized (data not shown) at 2 h after mMCP-5 recombinant protein injection ($2.3 \pm 0.2 \times 10^6$ cells/mouse) when compared with the number of cells recovered from control mice injected with control conditioned media ($1.7 \pm 0.1 \times 10^6$ cells/mouse) or with PBS ($1.5 \pm 0.3 \times 10^6$ cells/mouse). In these experiments, eosinophils increased from $0.25 \pm 0.13 \times 10^5$ of the total cells in the peritoneal exudate of control conditioned media–injected mice or PBS-treated littermates to $2.1 \pm 0.6 \times 10^5$ in mMCP-5–containing conditioned media–injected mice.

The injection of mMCP-5 induced an increase (~ 1.4-fold) in peritoneal lymphocytes that was not observed in control conditioned media–injected mice (Fig. 3). mMCP-5 injection resulted in an increase in peritoneal lymphocyte numbers caused by the predominant recruitment of B220$^+$ B lymphocytes ($5.7 \pm 0.4 \times 10^5$ cell/mouse in mMCP-5–injected mice versus $4.4 \pm 0.1 \times 10^5$ cell/mouse in control conditioned media–injected mice or $4.1 \pm 0.2 \times 10^5$ cell/mouse in PBS-treated mice; Fig. 3). The number of Thy1.2$^+$ T lymphocytes remained comparable in these three different groups of experimental mice at the same time point indicated ($5.8 \pm 2.3 \times 10^4$; $4.9 \pm 1.5 \times 10^4$; and $5.1 \pm 1.2 \times 10^4$ in mMCP-5–injected mice, control conditioned media–injected mice, and PBS-treated mice, respectively; Fig. 3).

No significant increases in the total number of neutrophils or macrophages recovered from the exudates of mMCP-5–injected mice were detected at any time point analyzed (Fig. 3 and data not shown). In contrast, there were twice as many monocytes 2 h after mMCP-5 injection compared
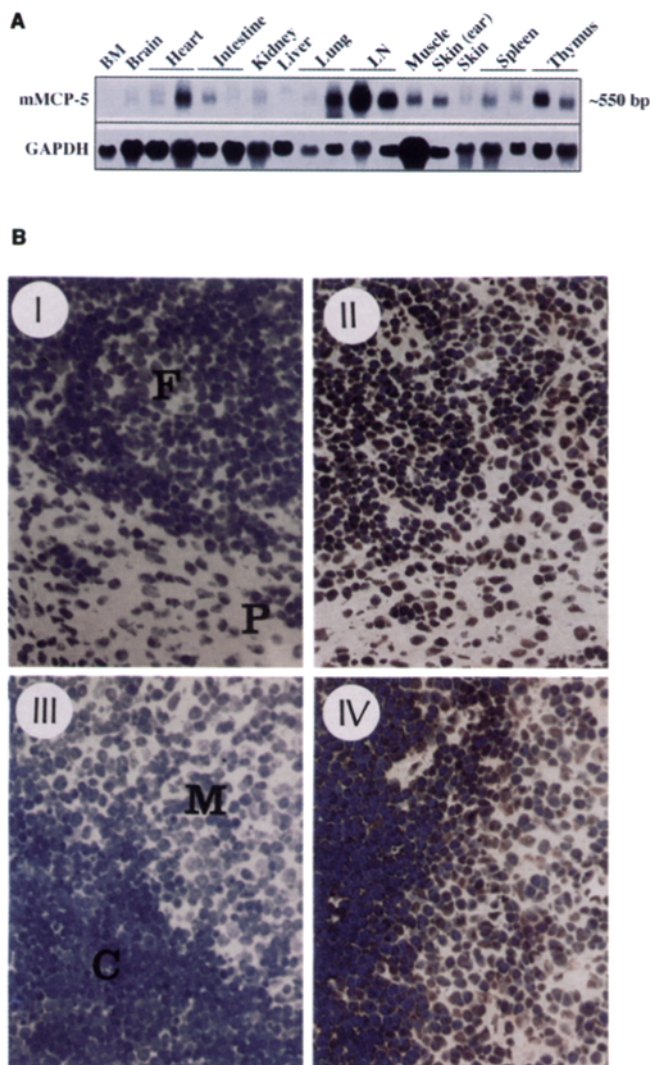
A

B

Figure 4. Expression of mMCP-5 under noninflammatory conditions. (*A*) Northern blot mMCP-5 cDNA with RNA from different mouse organs. For confirmation of mMCP-5 expression in some organs, two separately prepared RNAs from different mice were included in the Northern blot. The size of the hybridizing band is ~0.5 kb. (*B*) Immunohistochemical analysis of mMCP-5 expression in lymphoid organs. Inguinal LN (*I* and *II*) and thymi (*III* and *IV*) from nonimmunized mice were stained with anti–mMCP-5 mAbs (*II* and *IV*) or with an irrelevant isotype-matched negative control mAb (*I* and *III*). ×400. *C*, cortex; *M*, medulla; *P*, paracortex; *F*, follicle.

with that recovered from the exudate of mice injected with control conditioned media (Fig. 3).

Pattern of Expression of mMCP-5 under Noninflammatory Conditions. Only the LN and thymus expressed consistently high levels of mMCP-5 mRNA (~550 bp; Fig. 4 *A*). mMCP-5 mRNA were also found in heart, skin, lung, and spleen by RT-PCR (Fig. 4 *A* and data not shown), but no mMCP-5 mRNA was found constitutively in any other tissues of C57BL/6 mice (Fig. 4 *A*).

To determine the distribution of mMCP-5 protein in the LN and thymus, immunohistochemical analysis of sections from these organs was performed after staining with anti–mMCP-5 antibodies. In the LN, mMCP-5 staining

was generally localized within stromal-type cells in the paracortex and primary follicles (Fig. 4 *B*, *II*). Few, if any, lymphocytes or dendritic cells showed immunoreactivity (Fig. 4 *B* and not shown). In the thymus, stromal cells predominantly present in the medulla reacted to the mMCP-5 antibodies (Fig. 4 *B*, *IV*). A few cells in the cortex were stained, and there seemed to be no immunoreactive thymocytes present (Fig. 4 *B* and data not shown). No immunoreactivity to any cell type was recognized in either tissue using other irrelevant isotype-matched mAbs from the same hybridoma fusion (Fig. 4 *B*, *I* and *III*, and data not shown).

Expression and Function of mMCP-5 in the Lung during Allergic Inflammation. Northern analysis showed that mMCP-5 mRNA is markedly increased in the lungs of mice during the course of experimental lung eosinophilia (Fig. 5 *A*), using repeated doses of aerosolized OVA for 21 d (10). mMCP-5 mRNA expression, which was expressed at very low levels in the lung of unmanipulated mice, peaked 3 h after OVA challenge on the days analyzed (Fig. 5 *A* and data not shown), thus coinciding with the time point of maximal eosinophil accumulation on these days (10). Furthermore, mMCP-5 protein was localized by immunostaining to alveolar macrophages and to smooth muscle cells most strongly (Fig. 5 *B*). A small number of leukocytes within the large perivascular and peribronchiolar infiltrates showed low immunoreactivity (Fig. 5 *B* and data not shown). In areas of leukocyte localization in the lung tissue, there was an increase in reactivities of macrophages, smooth muscle cells, and other tissue resident cells that correlated with the severity of the infiltrate (not shown).

To evaluate the contribution of mMCP-5 to the development of OVA-induced lung eosinophilia, anti–mMCP-5 neutralizing Abs were administered 30 min before OVA challenge at the time of maximal eosinophil accumulation (days 20 and 21 of treatment). This antibody preparation was shown to block the transmigration of eosinophils to mMCP-5 in vitro (Fig. 2 *B*). Our results showed that blocking of mMCP-5 in vivo reduced by ~25% the number of eosinophils that accumulated in the BAL after OVA challenge at the same time point indicated (Fig. 5 *C*).

Regulation of mMCP-5 Expression In Vivo and In Vitro. Based on our previous finding (11) that the presence of lymphocytes is absolutely required for eosinophil accumulation in this model, we studied the regulation of mMCP-5 mRNA expression in the lungs of lymphocyte-deficient RAG-1 mutant mice (24). Fig. 6 *A* shows that there was strong reduction in the levels of mMCP-5 mRNA on days 15 and 18 in OVA-treated RAG-1 mice when compared with OVA-treated wild-type controls. It also revealed on day 21 a moderate, but notable, reduction in the level of expression of mMCP-5 in the lungs of OVA-treated RAG-1–deficient mice (*n* = 3; Fig. 6 *A*). In contrast, a comparable level of mRNA expression of Eotaxin, RANTES, and MIP-1α was found in the lung of mutant and wild-type control mice at each point during the OVA treatment (Fig. 6 *A*).

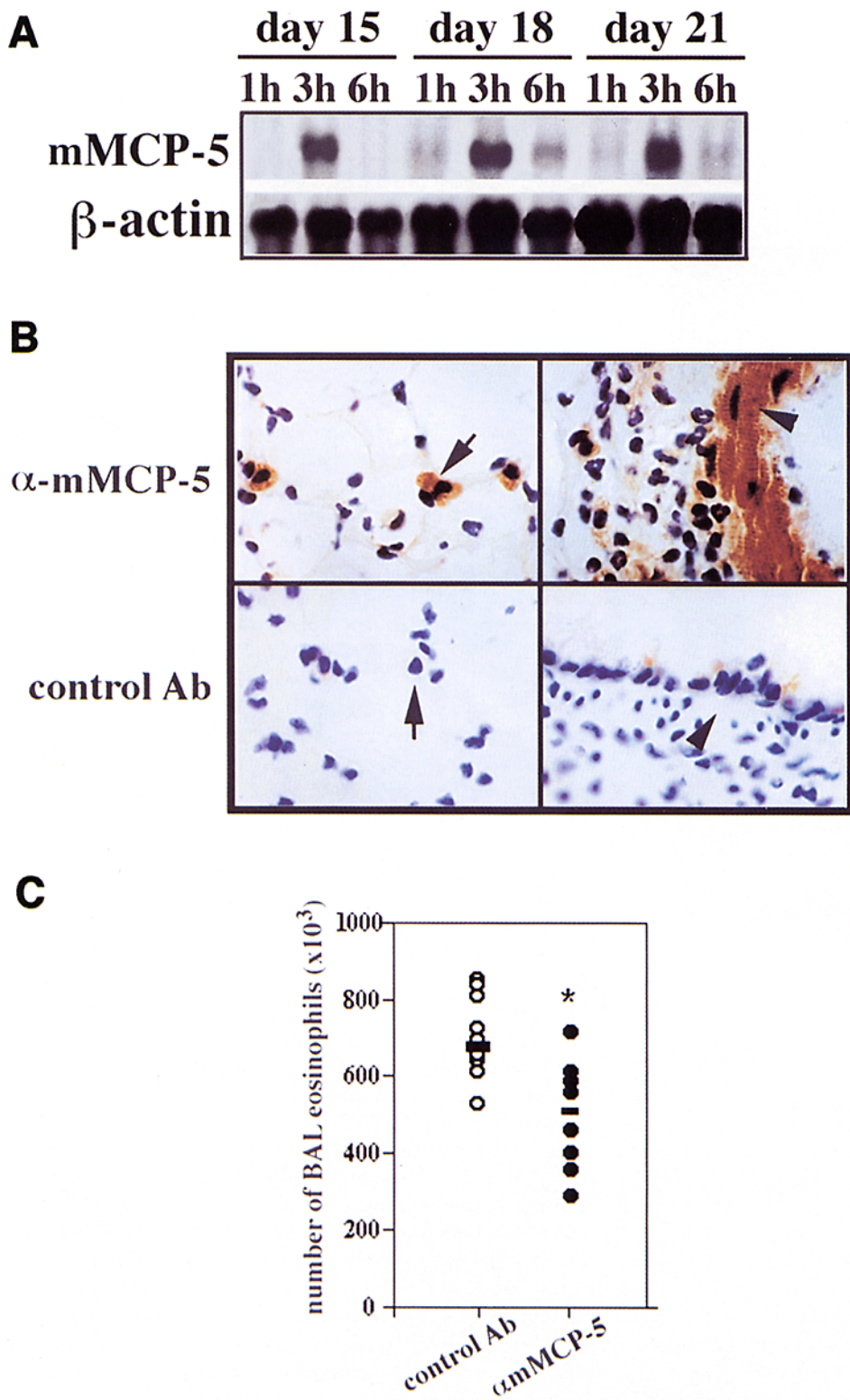To investigate which lymphocyte subtype(s) could be involved in the regulation of mMCP-5 expression, North-

A

day 15   day 18   day 21
1h 3h 6h 1h 3h 6h 1h 3h 6h

mMCP-5

β-actin

B

α-mMCP-5

control Ab

Figure 5. Expression and function of mMCP-5 mRNA during OVA-induced lung allergic inflammation. (*A*) At the different time points indicated after OVA administration, Northern blot analysis was performed to determine the expression of chemokines in the lung. (*B*) Expression of mMCP-5 protein in lungs from OVA-treated mice. Immunohistochemical analysis was performed upon lung tissue isolated on day 21 of OVA treatment using monoclonal anti–mMCP-5 (see Materials and Methods). Alveolar macrophages were prominently stained in OVA-treated mice (*arrows*), and smooth muscle cells (*arrowheads*) of the bronchioles were also densely stained in treated but not untreated mice. (×1,000). (*C*) Blocking of mMCP-5 protein with specific neutralizing antibodies (as used in Fig. 2 *B*) during the course of OVA-induced allergic inflammation (see Materials and Methods). Bronchoalveolar lavage was recovered from a treated mouse, and eosinophils were enumerated. Each dot represents a single mouse.

C

ern analysis was performed using RNAs from the lungs of mice lacking either T and NK cells (CD3ε-transgenic mice; 14) or CD4+ T cells (CD4-deficient mice; 25) or CD8+ T cells (CD8-deficient mice; 26) after OVA treatment. We found a comparable level of mMCP-5 mRNA expression in the lungs of these immunodeficient mice

when compared with that observed in wild-type littermates during OVA treatment (Fig. 6 *A* and data not shown).

To address the regulation of mMCP-5 expression in different lung resident cell types present during allergic eosinophilia, we evaluated the presence of mRNA for mMCP-5 in a panel of unstimulated and stimulated cell lines and in

freshly isolated cells of different lineages by RT-PCR (Fig. 6 B) or Northern blotting (data not shown). We compared this regulation with that of Eotaxin, whose strong and inducible mRNA expression was found not to be reduced in lymphocyte-deficient mice during OVA treatment (Fig. 6 A).

No expression of mMCP-5 mRNA was found on freshly isolated peritoneal macrophages by RT-PCR (Fig. 6 B). When macrophages were stimulated in vitro with IFN-γ, but not with TNF-α, however, they expressed mMCP-5 mRNA at an easily detectable level (Fig. 6 B). Conversely, the same population of freshly isolated peritoneal macrophages stimulated with TNF-α, but not with IFN-γ, expressed mRNA for mEotaxin (Fig. 6 B). Similar results were obtained when two macrophage cell lines were analyzed (Fig. 6 B). mMCP-5 mRNA expression was found in unstimulated or LPS-stimulated P388D1 macrophages, but not in RAW287 cells (Fig. 6 B). In contrast, only RAW287 cells expressed detectable mRNA levels of mEotaxin.
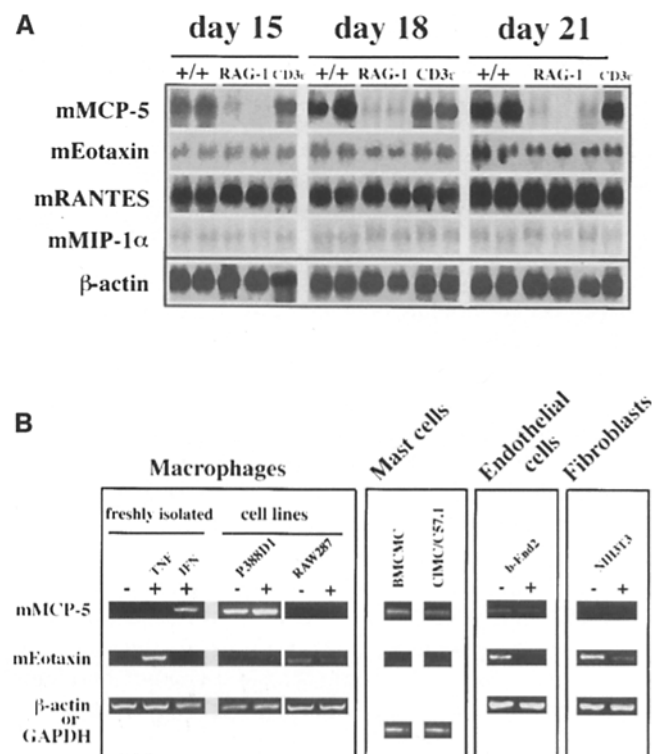
Figure 6. Regulation of mMCP-5 mRNA expression in vivo and in vitro (A) Expression of mMCP-5 mRNA in the lungs of RAG-1-deficient mice and CD3ε-transgenic mice during lung allergic inflammation. Northern blot analysis was performed using total RNAs from lungs 3 h after OVA administration on days 15, 18, and 21 of the treatment Expression of two representative wild-type mice out of five at each time point, two out of five RAG-1-deficient mice on days 15 and 18, and three out of five RAG-1-deficient mice on day 21, as well as one CD3ε-transgenic mouse on days 15 and 21, and two on day 18 is shown. (B) Comparison of the mRNA expression pattern of the mMCP-5 and mEotaxin genes in different cell lines and freshly isolated cells. Total RNA from cells indicated either without stimulation (−) or after exposure to different stimuli (+, see Materials and Methods) was amplified by RT-PCR with individual gene-specific primers. Either β-actin or GAPDH was used as the control for these experiments.

mMCP-5 mRNA was also found to be expressed by BMCMC and in the mast cell line ClMC/C57.1. In contrast, no mEotaxin mRNA was found in these two mast cell populations, either resting (Fig. 6 B and reference 10) or after stimulation with IgE and antigen (data not shown).

Low but detectable levels of mMCP-5 mRNA were observed in the endothelial cell line b-End2 with or without stimuli (Fig. 6 B), whereas no mMCP-5 mRNA was detected in nonstimulated or LPS-stimulated NIH3T3 fibroblasts (Fig. 6 B). In contrast, both cell lines express detectable levels of mEotaxin mRNA.

Role of mMCP5 in mEotaxin-induced Eosinophil Recruitment In Vivo and In Vitro. To examine whether the presence of mMCP-5 in the eosinophilic lung, in which Eotaxin is abundantly expressed, could lead either to a larger accumulation of eosinophils or is a possible mechanism to control the inflammatory response, Eotaxin (0.5 μg/mouse) was coinjected in the peritoneum either with 400 μl/mouse (Fig. 7 A) or 800 μl/mouse (data not shown) of conditioned media containing mMCP-5 recombinant protein or with the same volumes of the control conditioned media. The total number of peritoneal eosinophils recovered from mMCP-5–injected mice or from Eotaxin-injected mice 2 h after injection was $2.2 \pm 0.6 \times 10^5$ and $1.3 \pm 0.5 \times 10^5$ cells/mouse, respectively (Fig. 7 A). When both chemokines were administered simultaneously, the number of infiltrating eosinophils was lower ($0.8 \pm 0.3 \times 10^5$ cells/mouse, Fig. 7 A) than that found in the peritoneal exudate of mice injected with either one alone. In contrast, when mMCP-5 and mEotaxin were administered simultaneously at very low doses (100 and 0.2 μg/mouse, respectively), the number of infiltrating eosinophils ($0.5 \pm 0.18 \times 10^5$ cells/mouse) was greater than those found when mMCP-5 or mEotaxin were injected individually at the same doses ($0.09 \pm 0.01 \times 10^5$ cells/mouse for mMCP-5 and $0.2 \pm 0.03 \times 10^5$ cells/mouse for mEotaxin; Fig. 7 A).

To further define the chemotactic responses of eosinophils to combinations of mMCP-5 and mEotaxin, we examined them in vitro. The results from two individual experiments representative of this experimental series ($n = 5$) are shown in Fig. 7 B. mMCP-5 enhanced the migration of eosinophils to mEotaxin (from 3 to 10% in the presence of different concentration of mEotaxin). The maximum enhancement was reproducibly observed when 10 ng/ml of mEotaxin was combined with undiluted mMCP-5 conditioned media (Fig. 7 B). As the concentration of mEot was increased from 10 to 250 ng/ml in combination with undiluted mMCP-5–containing conditioned media in the different experiments, eosinophil migration was enhanced to a lesser extent, until no mMCP-5–induced enhancement of eosinophil chemotaxis was seen at 250 ng/ml of mEotaxin (Fig. 7 B). Furthermore, mMCP-5 reduced the mEotaxin-induced eosinophil transmigration in vitro when combined with a high concentration of mEotaxin (from 50 to 250 ng/ml, depending on the experiment). This effect was not observed when the control conditioned media was combined with different concentrations of mEotaxin in the same experiments (Fig. 7 B). We have selected two experi-
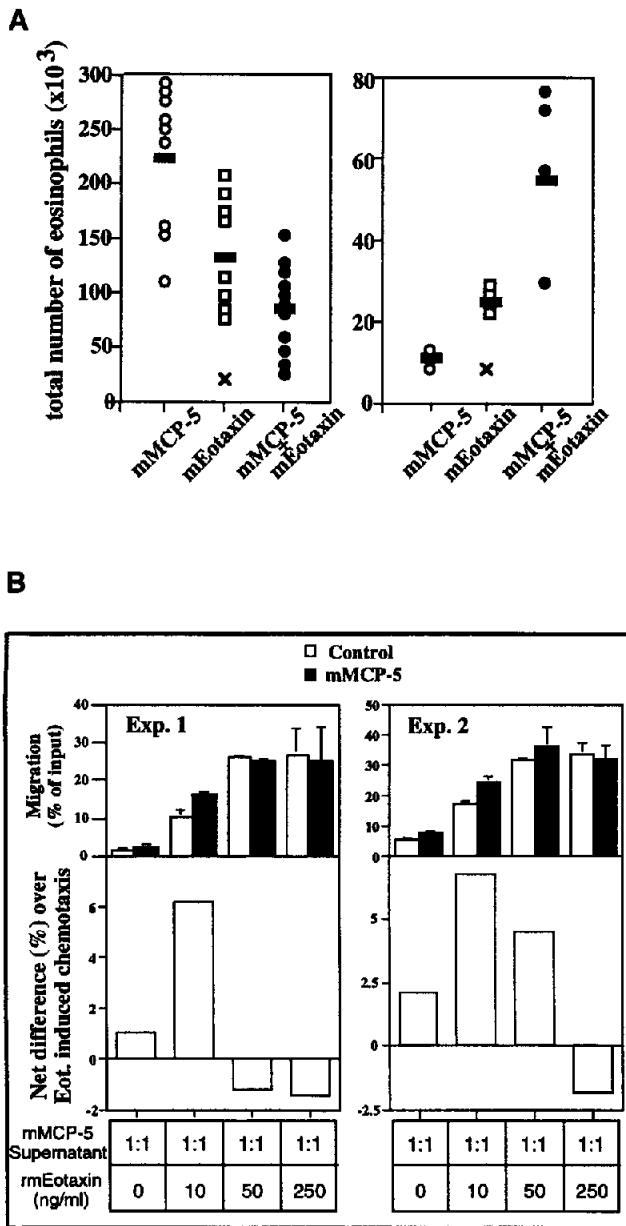
A

B

Figure 7. Effect of mMCP-5 on mEotaxin-induced eosinophil migration in vivo and in vitro. (*A*) Peritoneal eosinophil accumulation in response to mMCP-5 and mEotaxin coinjection. The number of eosinophils in the peritoneal exudate of C57BL/6J mice 2 h after injection with mMCP-5 (*open circles*), mEotaxin (*open squares*), or both chemokines simultaneously (*closed circles*) was determined as described in Materials and Methods. Values shown in the left panel reflect the number of eosinophils recovered from the peritoneal exudates of mice injected with 400 μl/mouse of mMCP-5–containing conditioned media and/or 0.5 μg/mouse of mEotaxin in 400 μl PBS, whereas values shown in the right panel are from mice injected with 100 μl/mouse of mMCP-5–containing conditioned media and/or 0 2 μg/mouse of mEotaxin in 400 μl of PBS. Control mice (*x*) injected with the same volume of control conditioned media and/or 400 μl/mouse of PBS (*n* = 4). Mice injected with Eotaxin (but not with MCP-5) were coinjected also with the same volume of control conditioned media. The bars in each panel represent the mean of the total number of eosinophils recovered from the mice analyzed (each symbol represents one individual mouse). (*B*) Eosinophil chemotaxis in response to combinations of mMCP-5 and mEotaxin in vitro in the transwell migration assay. Two independent experiments out of five are shown. The

percentage of input cells that migrated was measured. Each experimental point was tested in duplicate. As a control, the undiluted mock-conditioned media was mixed with different concentrations of mEotaxin in parallel and used in the same assays. The upper panel represents the raw data of two representative experiments out of five that were performed. Migration is expressed as mean values and bars show duplicates. The lower panel represents the net difference between the migration (percentage of input) of eosinophils to different concentrations of mEotaxin in the presence or absence of mMCP-5 (as shown in the upper panel). The values were calculated by subtracting (individually for each concentration of Eotaxin used) the percentage of eosinophil migration induced by mEotaxin in the absence of mMCP-5 (but with the same volume of control conditioned media) from the percentage of eosinophil migration induced by mEotaxin in the presence of mMCP-5.

ments to illustrate that we did observe donor-to-donor variation in the relative responses to mMCP-5 and mEotaxin, although the pattern discussed above was maintained.

Discussion

We have identified a novel mouse CC chemokine that is chemotactic for eosinophils, monocytes, and lymphocytes from the lung of mice during an allergic inflammatory response characterized by eosinophilic infiltration. Because of its polyspecificity, its differential gene regulation, and its modulatory effect on action of Eotaxin, we propose that mMCP-5 plays a pivotal role during lung allergic inflammation.

mMCP-5 Is a Novel C-C Chemokine Closely related to Human MCP-1. The amino acid sequence of mMCP-5 is very similar to that of members of the human MCP subbranch (27). The first amino acid in the mature protein that has been shown to be critical for MCP functions (28) is not conserved between mMCP-5 and hMCPs. Moreover, compared to three human MCPs, mMCP-5 has the longer peptide and has an additional cysteine at the COOH terminus, which might form an additional disulphide bond in the protein. It also has a lower theoretical pI than that of MCPs (27). mMCP-5 is very similar to hMCP-1 (65%), yet is quite different from mMCP-1/JE (45%) and from the recently described hMCP-4 (~51%; Fig. 1). Three reasons lead us to postulate that mMCP-5 is not the mouse homologue of hMCP-1: (*a*) the moderate monocyte chemotactic activity of low concentrations of mMCP-5, and its potent activity on eosinophils, which is different from the activities reported for hMCP-1 (29); (*b*) the observation that in a cross-species Southern blot with hMCP-1 and mMCP-1 probes, the same band is recognized (Rollins, B., personal communication); and (*c*) our preliminary observation that under conditions of high stringency, a single band is detected when an mMCP-5 probe is hybridized to a Southern blot containing human genomic DNA (experiments are underway in our laboratory to clone the full-length cDNA of this putative mMCP-5 homologue in humans).

mMCP-5 Is a Chemotactic Factor for Monocytes, Lymphocytes, and Eosinophils In Vitro and In Vivo. In this study, we demonstrate that mMCP-5 induces the chemotactic transmigration of monocytes, lymphocytes, and eosinophils in

vitro, but that it has no effect on the chemotaxis of neu-trophils (Fig. 2 A). While mMCP-5 induced significant chemotaxis of these three leukocyte subsets, eosinophils exhibited the greatest response. This response could be completely blocked by antibodies against mMCP-5. Al-though the migratory response to mMCP-5 in monocyte or lymphocyte populations was low (1.5–2%), the chemo-tactic index of these cell types was comparable to that found in these cell types to other chemoattractants, such as MIP-1α or RANTES (30). Among lymphocytes, B cells responded to mMCP-5 better than T cells did. mMCP-5 is the only mouse CC chemokine reported to date that promotes the chemotactic migration of B lymphocytes, although hMIP-1α has been reported to be chemotactic for human B lympho-cytes (31). The differential response of lymphocytes from different hemotapoietic organs to mMCP-5 is intriguing, and may reflect intrinsic differences in the migratory be-havior of lymphocytes from distinct locations. In vivo stud-ies showed that mMCP-5 was also effective in recruiting leukocytes to the peritoneum after challenge (Fig. 3).

Stromal Cells in the LN and Thymus Express Constitutively mMCP-5. The LN is a highly organized lymphoid tissue in which different leukocyte subsets and stromal cells ar-range themselves into specialized compartments to which particular subsets of lymphocytes migrate in a very specific fashion (32, 33). In the thymus, mature and immature thy-mocytes interact with stromal cells, and these interactions are critical for their maturation. The constitutive mRNA expression of mMCP-5 in the LN and thymus (Fig. 4 B), together with its chemotactic activity on lymphocytes, make it an interesting candidate for participation in this process. mMCP-5 does not support T nor B cell prolifera-tion in vitro (data not shown). It is notable that we de-tected mRNA for mMCP-5 in both skeletal and cardiac muscles (Fig. 4 A). We also found expression in heart, lung, and skin, but there was great variability between the mice that were studied.

mMCP-5 Is Different from the Other Major CC Chemokines in That Its Expression during Allergic Inflammation Is Critically Regulated by Lymphocytes. We show here that the expres-sion of mMCP-5 mRNA in the lung is maximal 3 h after OVA challenge on all days analyzed during OVA treatment (days 15, 18, and 21). However, a clear increase in mMCP-5 mRNA levels was not seen as treatment progressed. In-stead, the levels for mMCP-5 mRNA were almost constant 3 h after challenge at all time points analyzed (Fig. 5 A), contrasting with other CC chemokines in this model (10). The pattern of mRNA mMCP-5 expression might reflect: (a) its polyspecificity, and thus it is conceivable that this chemokine could be involved in the recruitment of mono-cytes (which accumulate in the lung at maximum levels on day 15) and of eosinophils and/or lymphocytes (which ac-cumulate in the lung at maximum levels on day 21) during the allergic process induced by the OVA treatment (10); or (b) different activation signals inducing chemokine expres-sion in different cell types, for example, in mast cells, mMCP-5 is readily expressed, whereas Eotaxin is not. Blocking the mMCP-5 protein with specific antibodies in

vivo during the induction of lung eosinophilia resulted in a significant decrease (~25%, P <0.05) in the number of eosinophils that accumulated in the lung. These results in-dicate that mMCP-5 contributes either directly or indi-rectly to the development of lung eosinophilia in this model. Although other eosinophilic chemokines are ex-pressed during the course of the OVA treatment (Fig. 6 A), mMCP-5 and RANTES are the predominant ones at ear-lier points of this particular inflammatory response (day 15). Accordingly, on day 15 of the OVA treatment, the block-age of mMCP-5 with Ab revealed that at this time point, this chemokine is responsible for 80% of the accumulation of eosinophils in the lung (Gonzalo, J.A., and J.C. Gutier-rez-Ramos, unpublished data).

Analysis of lung tissue from mice that were exposed to OVA (day 21, 3 h after challenge) revealed intense staining of alveolar macrophages and smooth muscle cells (Fig. 5 B) and much less intense staining of epithelial and possibly en-dothelial cells and fibroblasts (Fig. 5 B), which are well characterized for their ability to produce other chemokines, such as MCP-1 and MIP-1 (34). It is notable, however, that the cell type primarily responsible for Eotaxin produc-tion is the alveolar epithelium (10, 11). We failed to deter-mine by immunostaining if mast cells have MCP-5 immu-noreactivity in vivo.

A hallmark of mMCP-5, which distinguishes it from the other CC chemokines present in the eosinophilic lung, is its differential mRNA regulation during lung allergic inflam-mation. Only mMCP-5 mRNA expression was signifi-cantly reduced in the lung of OVA-treated, RAG-1 mu-tant mice (Fig. 6 A). This suggests that lymphocytes play an important role in the expression of mMCP-5 during aller-gic inflammation. Furthermore, similar levels of expression of mMCP-5 mRNA in the lungs of mutant mice lacking T cells (CD3ε-transgenic mice, CD4-deficient mice, and CD8-deficient mice) and wild-type littermates during OVA treatment demonstrate that B lymphocytes (RAG-1–deficient mice) but not T lymphocytes are essential for mMCP-5 expression (Fig. 6 A and data not shown).

It should be emphasized that no mMCP-5 immunoreac-tivity was found in infiltrating leukocytes in lung sections of OVA-treated mice (Fig. 5 B), and therefore it is unlikely that the strong reduction in mMCP-5 mRNA levels in the lung of OVA-treated, RAG-1–deficient mice could be at-tributed directly to the absence of few, if any, mMCP-5–expressing lymphocytes. Rather, we favor the hypothesis that B lymphocytes deliver key signals to induce mMCP5 expression by macrophages, smooth muscle cells, and/or other tissue resident cell types. This allows the hypothesis that regulation of the expression of mMCP-5 and of, for example, Eotaxin might be governed by different signals. Correspondingly, we found almost opposite patterns of ex-pression for Eotaxin and mMCP5 mRNAs after activation of primary cultures of macrophages or mast cells (mMCP-5 mRNA can be produced by the mast cells studied here, but not Eotaxin mRNA [Fig. 6 B and reference 10]). More-over, different mMCP-5 and mEotaxin mRNA expression was found in various cell lines representing some of the cell

lineages that may produce mMCP5 in the inflamed lung (Fig. 6 B).

The Migration of Eosinophils to Eotaxin Is Modulated by mMCP-5 In Vivo and In Vitro. Our results demonstrate that doses of either mMCP-5 or mEotaxin, which by themselves are each able to induce a significant increase in the number of infiltrating eosinophils, fail to exert the same effect when coinjected (Fig. 7 A). In contrast, doses of both chemokines that separately elicit a small but significant peritoneal recruitment of eosinophils showed a cooperative effect in the recruitment of eosinophils when simultaneously injected (Fig. 7 A). Also in vitro, our results showed that a constant amount of mMCP-5 can either enhance or reduce the chemotaxis of eosinophils to Eotaxin. Therefore,

mMCP-5 modified the bell-shape curve of the response to Eotaxin (Fig. 7 B). These results suggest that mMCP-5 would cooperate with low amounts of Eotaxin in augmenting the directed movement of eosinophils in the chemotaxis assay. When a certain concentration of Eotaxin is reached in the assay, however, the presence of mMCP-5 reduces the response to Eotaxin. Experiments are underway to determine if MCP-5 and Eotaxin share a receptor on eosinophils.

Our initial work on the novel chemokine mMCP-5 has focused on the characterization of its possible function in allergic inflammatory reaction situations. The expression and function of mMCP-5 have unique characteristics during lung allergic inflammation that distinguish it from other CC chemokines studied.

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