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Mouse Monocyte-Derived Chemokine Is Involved in Airway Hyperreactivity and Lung Inflammation

Jose-Angel Gonzalo,1* Yang Pan,1† Clare M. Lloyd,1* Gui-Quan Jia,1* Gary Yu, † Barry Dussault,1* Christine A. Powers, ‡ Amanda E. I. Proudfoot, ‡ Anthony J. Coyle,* David Gearing, † and Jose-Carlos Gutierrez-Ramos2*

The cloning, expression, and function of the murine (m) homologue of human (h) monocyte-derived chemokine (MDC) is reported here. Like hMDC, mMDC is able to elicit the chemotactic migration in vitro of activated lymphocytes and monocytes. Among activated lymphocytes, Th2 cells were induced to migrate most efficiently. mMDC mRNA and protein expression is modulated during the course of an allergic reaction in the lung. Neutralization of mMDC with specific Abs in a model of lung inflammation resulted in prevention of airway hyperreactivity and significant reduction of eosinophils in the lung interstitium but not in the airway lumen. These data suggest that mMDC is essential in the transit/retention of leukocytes in the lung tissue rather than in their extravasation from the blood vessel or during their transepithelial migration into the airways. These results also highlight the relevance of factors, such as mMDC, that regulate the migration and accumulation of leukocytes within the tissue during the development of the key physiological endpoint of asthma, airway hyperreactivity. The Journal of Immunology, 1999, 163: 403–411.

Chemokines are a group of structurally and functionally related cytokines able to induce a variety of functions on hematopoietic and nonhematopoietic cells by interacting with their specific receptors (1–4). Chemokines are involved in hematopoiesis, leukocyte exocytosis, and leukocyte trafficking (5–7). This multifunctional activity results in the recruitment of leukocytes to sites of inflammation and in the secretion of membrane products and inflammatory mediators that influence tissue damage (8–11). Because chemokines are specific for particular leukocyte subsets, the selective recruitment of leukocytes to sites of inflammation is strongly directed by these molecules (4).

Over the last few years, inflammatory reactions mediating allergic lung disease have been extensively studied regarding chemokine action (12–16). In fact, several chemokines have been identified in the inflamed lung of humans, mice, and guinea pigs and have been shown to be critically active during lung allergic processes (12, 17–23). These chemokines direct the recruitment of leukocyte types such as eosinophils, lymphocytes, and monocytes that invade the asthmatic lungs (12, 14, 22, 24–26). Chemokines also deliver signals that are involved in airway hyperreactivity (AHR),3 and it is possible to influence this physiological response by neutralizing chemokine signals at different time points during the response (27). The expression of several chemokines during lung allergic inflammatory responses is not necessarily redundant but potentially interdependent (27).

In this report, the mouse homologue of human MDC (hMDC) is described. hMDC, also described as stimulated T cell chemotactic protein-1 (STCP-1) (28), has been shown to be a potent monocyte, stimulated T lymphocyte, and activated Th2 lymphocyte chemoattractant that binds the chemokine receptor CCR4 (28–32). Here we show that murine MDC (mMDC), the expression of which is highly regulated during the course of an allergic inflammatory reaction in the lung, is produced by alveolar macrophages and smooth muscle cells, and the peptide encoded by this gene has chemoattractant activity on monocytes and stimulated lymphocytes in vitro. Neutralization of mMDC with specific Abs prevented interstitial lung inflammation and development of AHR in a murine model of lung inflammation.

Materials and Methods

Sequence database search

Sequences of known human chemokines were used to search public databases with the BLAST (basic local alignment search tool) algorithm (33). One murine clone, from the public database DBEST, containing hMDC and thymus and activation-regulated chemokine (TARC) homologous sequence (GenBank accession no. AA175762) (29, 34) was obtained from Research Genetics (Huntsville, AL) and fully sequenced. It was designated mMDC (see Results).

Cloning of mMDC and protein production

The full-length mMDC cDNA was cloned into mammalian expression vector pN8/e (a gift from Dr. J. Morgenstern, Millennium Pharmaceuticals, Cambridge, MA) by PCR. The coding region was amplified using the following primers: 3’ end primer, 5’-CGGAATTCCTAGTGAGTTTTACACAGTTGTCGCCAAAATGAGTTTGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCACCAGCTATGCTACCTGTGTTTGTGATTCTGCACGCCAAGGACTGTCACGCATCCAC...
washing with 25 mM imidazole, bound C-terminal-6xHis-tagged mMDC protein was eluted with 250 mM imidazole. Fractions containing recombinant mMDC protein were pooled and dialyzed against PBS.

**Anti-mMDC Ab generation**

Rabbit polyclonal Abs against murine mMDC were prepared according to standard methods (35). This Ab was generated against a 15-aa peptide corresponding to the N-terminal region of mMDC. The sequence of the peptide was GYPYAMEDSVCCRD. Rabbit serum was first depleted of anti-human IgG Abs by passage over a human IgG column, and anti-mMDC Abs were purified from the flow-through on an affinity column using the same peptide or using mMDC recombinant protein depending on the preparation (Research Genetics, Huntsville, AL).

**Mice and in vivo procedures**

C57BL/6J mice 8–10 wk old were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in the Millennium Pharmaceuticals, Inc., specific pathogen-free mouse facility. The mouse model of lung inflammation used here consists of two phases: sensitization (OVA, 0.1 mg/mouse i.p. on day 0; Sigma, St. Louis, MO) and induction of the response (2% OVA for 5 min intranasally (i.n.) on day 8 and 1% OVA for 20 min i.n. on days 15–21) (19) (Fig. 4A). PBS (i.p. and/or i.n.) was administered to mice as a negative control. For the blocking experiments, mice also received 20 μg/ mouse of neutralizing polyclonal Abs against mMDC. This Ab was administered i.v. 30 min before OVA provocation either on days 8 and 15 or on days 8 and 15–21 (Fig. 4A). OVA-treated control mice were injected with the same volume of rabbit Ig control Ab (Rh Ig) at the same time points indicated during treatment (Dako, Carpenteria, CA). Three hours after OVA administration on day 15 or day 21, mice were sacrificed by CO2 asphyxiation and analyzed for lung inflammation and AHR. Bronchoalveolar lavage (BAL) was performed as described (19). AHR was expressed as enhanced pause (Penh), a calculated value, which correlates with measures of airflow resistance, impedance, and intrapleural pressure in the same mouse (36): \[ Penh = \frac{TW - TL}{P_{Fe}P_{Ffr}} \] (TL, expiration time; Tr, relaxation time; Pfe, peak expiratory flow; Pff, peak inspiratory flow × 0.67 = coefficient). The relaxation time is the time it takes for the box pressure to change from a maximum to a user-defined percentage of the maximum. Here, Tr measurement begins at the maximum box pressure and ends at 40%. AHR was measured 3 h after the last Ag challenge by recording respiratory pressure curves by whole-body plethysmography (Buxco Electronics, Sharon, CT) in response to inhaled methacholine (Mch; Aldrich Chemical, Milwaukee, WI) as described (27).

Peritoneal recruitment assays in vivo with mMDC protein were performed after injection of 800 μl i.p. of mMDC recombinant protein-containing conditioned medium or control conditioned medium. At different time points after injection (0, 1, 2, 4, and 6 h), peritoneal leukocytes were collected and analyzed. In one series of blocking experiments, mice were injected i.v. either with 20 μg/mouse of anti-mMDC neutralizing Ab or with Ab control, 30 min before mMDC recombinant protein-containing conditioned medium. Peritoneal lavage was performed 2 h after chemokine injection.

**Immunohistochemical phenotyping and quantitation of leukocytes**

Total BAL cell and peritoneal cell counts were performed, and aliquots (5 × 105 cells/slide) were pelleted onto glass slides by cytocentrifugation. To determine the number of eosinophils and neutrophils, slides were stained with Wright-Giemsa stain (Fisher Diagnostics, Pittsburgh, PA). T lymphocytes, B lymphocytes, and macrophages were identified by Thy 1.2 (53-21, 53-21) (PharMingen), and Moma 2 (BioSource International, Camarillo, CA) staining, respectively, as described (14). Percentage of eosinophils, lymphocytes, neutrophils, and macrophages was determined by counting their number in eight high power fields (×40 magnification; total area, 0.5 mm²) per area randomly selected and dividing this number by the total number of cells recovered from the BAL fluid. To obtain the total number of each leukocyte subtype in the lavage, these percentages were multiplied by the total number of cells recovered from the BAL fluid.

Lung sections from the different experimental groups of mice were prepared as described (14). Briefly, lungs were fixed in 10% neutral buffered formalin (J.T. Baker, Phillipsburg, NJ) and paraffin embedded. Sections (4 μm) were stained with hematoxylin and eosin according to standard protocols. An estimation of the percentage of each leukocyte subtype within the infiltrate in OVA + anti-mMDC Ab-treated mice or OVA + rabbit Ig-treated controls was made by counting 200 cells in one randomly selected peribronchial infiltrate and determining the number of eosinophils. Quantitation of leukocytes both in BAL fluid and in lung sections was performed in a blinded fashion.

**In vitro chemotaxis**

The in vitro migration of leukocytes to recombinant mMDC through an endothelial cell layer was evaluated in duplicate as described (19). The endothelial cells in transwell inserts were washed once with serum-free medium, and 2 × 10⁴ leukocytes (BM cells from C57BL/6J mice, Th1 or Th2 polarized lymphocytes, eosinophils from IL-5 transgenic mice (37), or Staphylococcus aureus enterotoxin B (SEB)-stimulated (10 μg/ml, 12 h) or unstimulated lymph node cells from C57BL/6J mice) were added in 0.1 ml of serum-free medium. After a 2-h incubation, the Transwells were removed and the number of cells per well was counted by the FACSScan by passing each sample for a constant predetermined time period (containing endothelial cells were gated out). In the blocking experiments, leukocytes were preincubated with either 1 or 10 μg of anti-mMDC Ab or control Ab at 37°C for 15 min before their addition to the Transwell inserts.

**Generation of the HEK/mCCR4 cell line**

Murine CCR4 was cloned from a mouse thymus cDNA library by PCR using primers based on the mouse CCR4 sequence (38). The full coding sequence of mCCR4 was subcloned into the mammalian cell expression vector pCDNA3.1.zeo (Invitrogen). Stable cell lines were generated following transfection of the expression vector into HEK-293 cells using the calcium phosphate transfection system (Life Technologies) according to the manufacturer’s instructions. Positive clones were selected with zeocin (100 μg/ml Invitrogen), and clones expressing high levels of mCCR4 were identified by binding to 3H-labeled human TARC (Amersham).

**Calcium mobilization**

The ability of mMDC to activate CCR4 was determined by adding 5, 25, and 50 aliquots of the conditioned medium containing mMDC to 1 × 10⁴ fura-2-loaded HEK/mCCR4 cells for each measurement, as previously described (39). Desensitization experiments were conducted by adding 50 μl of the conditioned medium containing mMDC, followed by the addition of different concentrations of human TARC or hMDC 60 s later.

**Th1-Th2 cell polarization**

Mice expressing the transgene for the DO11.10 αβ-TCR, which recognizes residues 323–339 of chicken OVA in association with I-AK (40), were provided by Dr. D. Loh (Washington University, St. Louis, MO). OVA-specific TCR-transgenic CD4+ T cells were isolated from the spleen (>97% purity) by using mouse CD4+ T cell subset enrichment columns (R&D Systems, Minneapolis, MN) and cultured in complete RPMI 1640 medium containing 10% OVA323–339 (1 μg/ml) and mitomycin C-treated splenocytes. For Th1 phenotype development, recombinant murine IL-12 (40 ng/ml) (Endogen, Cambridge, MA) and neutralizing anti-IL-4 Ab (11B11, 20 μg/ml, R&D Systems) were added and for Th2 phenotype development recombinant murine IL-4 (50 ng/ml and anti-IL-12 (TOS-2, 10 μg/ml, Endogen) were used. Cells were cultured for three rounds of antigenic stimulations under polarizing conditions. To determine that cells were differentiated, 2 × 10⁵ cells were activated on immobolized anti-CD3 mAb (2C11, 10 μg/ml, PharMingen) in the presence of human IL-2 (10 U/ml) (Endogen) for 48 h. IL-4, IL-5, and IFN-γ levels were determined by specific ELISA (Endogen). In general, Th2 cells produced high levels of IL-4 and IL-5 but little IFN-γ, whereas Th1 cells produced high levels of IFN-γ but little IL-4 and IL-5. 100% murine IL-4, IL-5, and IL-12 (TOS-2, 10 μg/ml, R&D Systems) cells produced 100–300 pg/ml/ml IL-4, 50–150 ng/ml IL-5, and <20 pg/ml IFN-γ. Th1 cells produced 7,000–15,000 ng/ml IFN-γ. The viability of Th1 and Th2 cells was >95%. Subsequently, these cells were used for both CCR4 expression analysis and in vitro migration assays.

**Measurement of mMDC protein by immunohistochemistry**

mMDC protein expression was determined in both normal and inflamed mouse lung tissue with a polyclonal rabbit anti-mMDC Ab using a modified avidin/biotin staining method as described (14). Sections were overnight with 20% normal donkey serum in PBS for 15 min and then incubated overnight at 4°C with anti-mMDC Ab diluted 1:750 in PBS with 0.1% BSA and 0.1% sodium azide. Endogenous peroxidase was subsequently blocked by incubation in methanol containing 0.3% hydrogen peroxide. Nonspecific staining due to cross-reaction with endogenous avidin or biotin was blocked by incubation with avidin solution followed by biotin solution, both for 20 min. Bound Ab was visualized by incubation...
with biotinylated anti-rabbit Ig diluted in 10% normal mouse serum PBS and then with streptavidin peroxidase complex prepared according to the manufacturer’s instructions (both from Dako) and incubated for 1 h each. Finally, slides were flooded with peroxidase substrate solution for 10 min before counterstaining with hematoxylin. Control slides with the following were included: 1) staining with normal rabbit Ig instead of primary Ab, 2) omission of biotinylated anti-rat Ig, and 3) omission of streptavidin complex. In addition, competitive inhibition of the Ab was accomplished by preincubation of Ab with the peptide (100-fold excess) for 45 min at 37°C before incubation with tissue sections.

**Measurement of mMDC mRNA expression**

mMDC mRNA expression in normal murine tissues was analyzed by Northern blot analysis. 

**Results and Discussion**

**Identification of mMDC gene**

Several mouse chemokine-like sequences were identified after searching the public database DBEST. One clone contained a novel full-length CC chemokine, which was designated mMDC based on its pattern of tissue expression, chemoketic specificity in vitro, and receptor usage. Nucleotide and amino acid sequence of this clone and homology analysis are not shown because, during the preparation of our manuscript, a novel mouse chemokine named ABCD-1 showing the same gene and protein sequence was reported (41).

The mouse chromosomal location of mMDC was determined by using a panel of backcross progeny of C57BL/6j Mus musculus and Mus spretus mice. The mapping results indicated that mMDC is located on mouse chromosome 2 (data not shown).

**Anti-mMDC polyclonal Ab reacting with recombinant protein**

Recombinant mMDC protein was produced in 293 EBNA cells by transient transfection as described in Materials and Methods. Purification of 6xHis-tagged mMDC protein was made following the Ni-NTA affinity purification method (Qiagen). Affinity-purified rabbit polyclonal Abs generated against a 15-aa peptide corresponding to the N-terminal region of mMDC were shown to recognize a specific band in a Western blot against mMDC. Using this polyclonal antisera, a single 8-kDa band was detected from the mMDC-transfected supernatant mentioned above but not in control supernatant (data not shown). This polyclonal Ab did not cross-react with murine macrophage-inflammatory protein-1α, murine monocyte chemoattractant protein (MCP)-1, mMCP-5, murine eotaxin, or stromal cell-derived factor -1α (SDF-1α) protein (data not shown). Furthermore, as shown later, in vitro leukocyte migration in response to either SDF-1α or MCP-5 was not affected by the anti-mMDC polyclonal Ab. Because it is known that in general the N-terminal regions of chemokines, such as IL-8 and MCP-1, contain receptor binding sites, it is likely that this Ab masked the receptor binding site on mMDC. This hypothesis is demonstrated below, where the neutralizing capabilities of the Ab preparation are shown.

**In vitro chemotactic responses of leukocytes to mMDC**

To evaluate the chemotactic function of mMDC on resting leukocytes, neutrophils, lymphocytes, and monocytes were isolated from C57BL/6j mouse bone marrow and subjected to in vitro transendothelial migration assays (42). Fig. 1A shows that the conditioned medium containing mMDC (but not the control conditioned medium) or purified mMDC protein induced the migration of monocytes. This migration was similar to that provoked by MCP-5 (Fig. 1A) and was three times smaller than that induced by SDF-1 (data not shown), which were used as positive controls. Neither bone marrow neutrophils from wild-type mice nor eosinophils from IL-5 transgenic mice (37) migrated in response to mMDC (data not shown). No effect on resting bone marrow lymphocytes in response to mMDC was detected in the same set of migration assays in which SDF-1 was the positive control (Fig. 1A). However, when lymph node T lymphocytes were stimulated in vitro with the superantigen *Staphylococcus aureus* enterotoxin B for 12 h (43), these cells were able to migrate in response to mMDC, showing a 3-fold increase in chemotactic index when compared with that observed in unstimulated cells (Fig. 1B). To further evaluate chemotactic function of mMDC on activated T lymphocyte subclasses, IL-2-stimulated Th1 and Th2 polarized cells were used in chemotaxis assays (Fig. 1B). Our data clearly showed that although mMDC induced both Th1 and Th2 cell migration, the chemotactic index of Th2 cells in response to mMDC was significantly higher than that observed in Th1 cells (Fig. 1B).

To attribute unambiguously the migration of monocytes and stimulated lymphocytes to the mMDC chemotactic activity within the serum-free supernatant from mMDC-transfected cells or to purified mMDC protein, this chemokine was blocked in vitro with affinity-purified polyclonal Abs raised against an mMDC peptide (see Materials and Methods). Specific anti-mMDC Ab was able to neutralize almost all of the monocyte and lymphocyte chemotactic activity of the purified mMDC protein or in the mMDC-conditioned medium (Fig. 1, A and B). Both in vitro chemotaxes induced by SDF-1α and by MCP-5 were unaffected by the anti-mMDC Ab (Fig. 1, A and B).

**In vivo chemotactic responses to mMDC**

To determine the efficacy of mMDC in vivo, serum-free supernatant from mMDC-transfected cells was injected into the peritoneum of mice. Maximal peritoneal leukocyte accumulation in response to mMDC was detected 2 h after injection (data not shown). At this time point, quantitation of leukocyte subtypes revealed 1-fold increase in monocyte numbers after mMDC injection (Fig. 1C). Peritoneal monocyte numbers (detected by Moma 2-positive cell staining) in the mMDC-treated mice and IL-8-treated control littermates were (215 ± 20) × 10³ and (140 ± 12) × 10³, respectively. This mMDC-induced monocyte accumulation in vivo correlates well with the in vitro data shown above. No differences in the total number of leukocytes or in the number of each cell type were detected when PBS-treated mice and control medium-injected mice were compared (Fig. 1C).

No discernible increase in peritoneal lymphocytes were detected following i.p. mMDC administration in the experimental group of mice when compared with PBS- or control medium-treated mice (Fig. 1C). Stimulated lymphocytes, but not resting lymphocytes, migrate in in vitro assays in response to mMDC (Fig. 1), suggesting that resting lymphocytes must be activated before responding to this chemokine and that mMDC is not able to mediate this activation.

The specificity of the migratory response to mMDC within the tissue culture supernatant injected was also confirmed in vivo by
using anti-mMDC neutralizing Ab. No monocyte accumulation was detected after coinjection of the mMDC protein-containing supernatant and the specific neutralizing Ab against this (Fig. 1C).

**Ca^{2+} flux in CCR4-transfектant cells and MDC cross-desensitization assays**

Taken together, the previous data indicate that mMDC displays chemotactic activities on monocytes and stimulated T lymphocytes. Because of the homology between mMDC and the human chemokine MDC (29), also described as STCP-1 (28), mMDC could be considered as the mouse homologue. In fact, like mMDC, hMDC is a potent chemoattractant for monocytes and stimulated T lymphocytes (28–30). In addition, hMDC has been described as a functional ligand for the chemokine receptor CCR4 (30). Corresponding to the highest expression of hCCR4 on Th2 lymphocytes vs Th1 lymphocytes, hMDC is much more active on Th2 cells than on Th1 cells (31). Similarly, mouse Th2-polarized lymphocytes, which also show higher levels of expression of CCR4 than Th1 cells by PCR (data not shown), respond more readily to mMDC in in vitro migration assays than do Th1 cells (Fig. 1B).

To confirm that mMDC utilizes the CCR4 receptor, calcium mobilization in response to mMDC was evaluated in HEK-293 cells transfected with mCCR4. Fig. 2 shows that calcium flux was induced in these cells following stimulation by conditioned medium containing mMDC. No calcium flux was detected when control conditioned medium or PBS in the peritoneum of the control littermates (open symbols). Thirty minutes before injection of mMDC-containing conditioned medium, experimental mice were injected i.v. with 20 μg/mouse of either anti-mMDC Ab (filled symbols) or Ab control (gray symbols). Each dot represents one individual mouse analyzed (5 mice per control group, 10 mice per experimental group). The bar in each panel represents the mean of the total number of cells of the leukocyte type indicated.
able to desensitize CCR4, because hMDC- or TARC-induced calcium flux in CCR4-transfectant cells was abolished in a dose-dependent manner by the murine chemokine (Fig. 2).

Regulation of mMDC expression during the course of lung inflammation

mMDC mRNA expression in normal murine tissues was examined by using several multiple-tissue Northern blots probed with the mMDC cDNA. As described for hMDC (28, 29), the greatest mMDC mRNA expression was detected in the thymus and lower in the lung (data not shown and Fig. 3A). No or little mMDC mRNA expression was detected in the spleen, brain, liver, or kidney (data not shown and Fig. 3A).

Because mMDC induces the migration of monocytes, activated T lymphocytes, and Th2 cells and these leukocyte types are critical players in the evolution of inflammation, the modulation of mMDC expression in pulmonary inflammation was examined. Therefore, mMDC mRNA expression was measured by RNase protection assay in lungs isolated at different time points during OVA-induced lung allergic inflammation (19). mMDC was expressed at low levels in the lung of PBS-treated mice (Fig. 3A) but was up-regulated by 5-fold by day 15 of OVA treatment (Fig. 3A). Expression peaked at this time point but did not return to basal levels. Interestingly, day 15 correlates with the peak accumulation of monocytes/macrophages in this model and precedes the accumulation of T lymphocytes and eosinophils (see below and Fig. 4). When the expression of mMDC was analyzed at late stages (day 18, day 21) of the inflammatory response in this mouse model, it was shown that mMDC mRNA was still up-regulated but to a lesser extent than at day 15 (Fig. 3A). Eotaxin expression at the same time points is shown for comparison (Fig. 3A).

Polyclonal Ab specific for mMDC was used to determine protein expression during allergic lung disease. Low, but detectable, mMDC protein expression was observed in the lungs of PBS-treated mice, but increased mMDC expression was detected in alveolar macrophages, infiltrating macrophages, and smooth muscle cells on days 15 and 21 of OVA-treated littermates. In addition, a subset of eosinophils, mainly those confined to the alveolar spaces, stained positive for mMDC (Fig. 3B). The phenotype of positive cells was determined by localization and morphology.

Blockage of mMDC during lung allergic inflammation

The inflammatory response to OVA in the mouse model studied here consists of a lung accumulation (interstitium and airway lumens) of macrophages that becomes maximal at early stages of the response (monitored here 3 h after OVA challenge on day 15) and an accumulation of eosinophils and lymphocytes that reaches its plateau at late stages of the response (monitored here 3 h after OVA challenge on day 21) (14) (Fig. 4). AHR is a feature of the late stages but not of the early stages in this specific model (27).

Role of mMDC at early stages of the response. Because mMDC mRNA expression is highly up-regulated at early stages of this pathological response (day 15) (Fig. 3), mMDC neutralization experiments were performed first at these time points (days 8 and 15) during OVA treatment (Fig. 4A). Thereafter, mice were analyzed on day 15, coinciding with maximal infiltration of monocytes and macrophages in the lung (14). Three hours after OVA challenge on day 15, the numbers of the different leukocyte types were analyzed both in the airway lumen (BAL fluid) (Fig. 4B) and in the lung interstitium (lung sections) (data not shown). The specific blockage of mMDC revealed a 44% decrease in the number of BAL monocytes in response to OVA at the time point indicated (Fig. 4B). Blockage of macrophage/monocyte activity in this model has been shown to affect eosinophil recruitment in the lung (27). Therefore, the concomitant 50% reduction in OVA-induced BAL eosinophilia after mMDC blockage was not entirely unexpected (Fig. 4B). Lymphocyte or macrophage accumulation in the BAL fluid was not significantly affected by the neutralization of the chemokine (Fig. 4B). A reduction in eosinophil and monocyte accumulation similar to that observed in the airway lumen was found in the lung interstitium of OVA-treated mice after mMDC neutralization (data not shown). Number and phenotype of bone marrow and spleen cells were not affected during the whole treatment by the anti-mMDC Ab when compared with control littermates (OVA or OVA + Ig control Ab) (data not shown). However, because at this time point the size of the infiltrate in the interstitium is small and the content of eosinophils is low, there is no significant AHR being induced. Therefore, we could not establish the impact of mMDC blockage in AHR.

Role of mMDC at late stages of the response. To study the influence of mMDC in lung inflammation and AHR at late stages of the inflammatory response, neutralizing Abs against mMDC were administered on days 8 and 15–21 (Fig. 4A). Mice were analyzed 3 h after OVA challenge on day 21, coinciding with maximal infiltration of the lung by eosinophils and T lymphocytes (14). OVA-induced leukocyte accumulation in the airway lumen (as detected in the BAL) in general, and eosinophil, lymphocyte, and monocyte in particular, was not affected at late stages of the inflammatory response by mMDC neutralization (Fig. 4C). Similarly, the neutralization of the monocyte, lymphocyte, and eosinophil chemokine macrophage-inflammatory protein-1e during the same mouse model of inflammation does not affect monocyte and lymphocyte infiltration in the lung (27), indicating that chemokine activity is strongly regulated in vivo and may not correspond with the expected activity based on in vitro assays. This may be explained by a dominant functional role played by other chemokines, such as eotaxin, RANTES, MCP-1, and MCP-5, that are expressed at this time in the lung (27). Chemokines have been shown to
coordinately activate different cellular and molecular pathways involved in the pathophysiology of asthma. Thus, MCP-1, which is not a predominantly lymphocytic or eosinophilic chemokine, diminishes both lymphocyte-derived inflammatory mediators and T cell and eosinophil recruitment to the lung of mice subjected to the same OVA model (27). Minimal OVA-induced eosinophil and T

FIGURE 3. mMDC mRNA and protein expression in mouse. A, mMDC mRNA expression by RPA in the lung of OVA-treated mice. Inflamed lungs were obtained from mice subjected to OVA treatment 3 h after Ag challenge at the time points indicated (days 0, 8, 15, 18, and 21). mMDC mRNA expression in normal and inflamed murine lung (first five lanes) and normal kidney (final lane) is shown (left). Results from using a GAPDH control probe are shown in the lower portion of the figure. Each full bar in the right panel represents the mean level of mMDC mRNA expression from five mice at the time points indicated (3 h after OVA challenge) during treatment. Eotaxin mRNA expression at the same time points is also shown for comparison (left panel; open bars in right panel). Values are expressed as fold increase in mMDC or eotaxin expression, respectively, over that in PBS-treated lungs (designated a value of 1). Although mMDC is more highly expressed than eotaxin at time 0, both chemokines were normalized to 1 to better illustrate the expression pattern during disease. Values are also expressed as mean + SEM. B, mMDC protein expression in the lung of OVA-treated mice. Sections were prepared from lungs isolated on day 15 (i), 21 (ii), or 0 (iii, left panel) of OVA treatment and were stained with a polyclonal Ab that recognizes mMDC. Positive staining was detected with an avidin-biotin peroxidase staining system that resulted in a brown reaction product. Sections were counterstained with hematoxylin (blue) for contrast. Protein expression was detected in macrophages (iv) and smooth muscle cells (vi) on day 15 as well as in a proportion of infiltrating eosinophils on day 21 (v) (as indicated by arrows). Preincubation of the anti-mMDC Ab with the immunizing peptide (iii, right panel) or irrelevant control Ab did not show any staining.
lymphocyte accumulation is detected in the lung interstitium after the neutralization of the monocytic chemokine MCP-5, whereas BAL eosinophil, monocyte, lymphocyte, and macrophage accumulation was evaluated 3 h after OVA administration on day 15 (B) or 21 (C). Each dot represents a single PBS or OVA + rabbit Ig control Ab (Rb Ig)-treated mouse (open symbol) or a single OVA + anti-mMDC Ab-treated mouse (filled symbol). Bars represent the mean of each group. One representative experiment of three, with 10 mice per group, is shown. Significant difference between control and test groups of mice was determined using the Student’s t test (p < 0.001).

FIGURE 4. Leukocyte accumulation in the airways after mMDC blockage during lung allergic inflammation. A, mMDC neutralization was performed daily before each aerosolized provocation with OVA either on days 8 and 15, or on days 8 and 15–21. BAL eosinophil, monocyte, lymphocyte, and macrophage accumulation was evaluated 3 h after OVA administration on day 15 (B) or 21 (C). Each dot represents a single PBS or OVA + rabbit Ig control Ab (Rb Ig)-treated mouse (open symbol) or a single OVA + anti-mMDC Ab-treated mouse (filled symbol). Bars represent the mean of each group. One representative experiment of three, with 10 mice per group, is shown. Significant difference between control and test groups of mice was determined using the Student’s t test (p < 0.001).

Role of mMDC in the induction of AHR

To determine whether the reduction of inflammation in the lung interstitium, but not in the airway lumen, following mMDC neutralization is associated with changes in airway function, AHR was evaluated in OVA-treated mice after mMDC blockage. Fig. 6
shows that mMDC neutralization inhibits the development of AHR in this experimental group of mice when compared with OVA control-treated mice. This decrease correlates with the 70% reduction in leukocyte accumulation, mainly eosinophils, in the lung interstitium of OVA-treated mice after mMDC blockage. This suggests a correlation between the location of the inflammatory cells in the lung and the establishment of AHR. Evidence indicates that eosinophils accumulated in the airways of asthmatic patients dictate the severity of disease (44). However, it has also been reported that the development of AHR depends on the recruitment of eosinophils to the mouse bronchial submucosa, but not to the airways (45). In addition, the blockage of the chemokine MCP-5, which affects interstitial eosinophil recruitment but not airway eosinophilia, abrogates AHR (27). Likewise, a recent clinical study has found no significant correlation between the degree of AHR and the number of inflammatory cells in sputum or BAL (46).

Concluding remarks

The murine model studied here has allowed us to dissect the predominant features of allergic lung disease, namely, cellular inflammation and bronchial hyperreactivity. Disease development necessitates the migration of leukocytes from the peripheral circulation through the vascular endothelium, across the lung interstitium, and into the airway lumen via the bronchial epithelium. We have previously determined that chemokines act in a tightly controlled, coordinated fashion to direct migration of leukocytes through the interstitium (as observed in histology) and into the airway lumen (as observed in lavage). The data presented here indicate that the presence of eosinophils (and, indeed, other inflammatory cells) in the airway lumen is not sufficient to induce the AHR that is characteristic of asthmatic processes. Rather, our data indicate that eosinophils and other inflammatory cells have to be localized within the peribronchial submucosa or in perivascular regions (as detected in sections of the lung interstitium) to induce these deleterious effects.

Our data support the notion that the chemokine mMDC is critical for the retention/trafficking of eosinophils and other leukocytes and for their proper localization to these areas in the lung during the development of an allergic reaction. During preparation of our manuscript, a mouse novel chemokine named ABCD-1 showing the same nucleotide and amino acid sequence was reported (41). This recent study describes ABCD-1 as the first activated T cell chemokine produced in large amounts by activated B cells. This suggests the possible implication of mMDC/STCP-1/ABCD-1 during a T cell-dependent B cell-humoral response.

The finding that mMDC is able to induce the recruitment of effector or regulator leukocytes during the allergic reaction makes the findings mentioned above even more relevant for the development of disease.

Finally, although there is no synteny between the chromosomal location for mMDC and hMDC loci (mouse chromosome 2 [data not shown] and human chromosome 16 [29] respectively), based on sequence homologies, pattern of tissue expression, receptor usage, and functional activities, mMDC may be considered the murine homologue of hMDC/STCP-1.

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References


FIGURE 6. Inhibition of OVA-induced AHR after mMDC blockage. Results are shown as the mean ± SEM for Penh before (open bars) and after (filled bars) Mch provocation (n = 10, three independent experiments). Mice were exposed to an aerosol of Mch for 1 min, and airway constriction was evaluated for the next 5 min. Mice treated with PBS or OVA + rabbit Ig were used as controls for OVA-treated littermates in which mMDC was blocked from days 8 to 21. Significant difference between control and test groups of mice was determined using the Student’s t test (p < 0.01) and is indicated by an asterisk.


