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Monocyte Chemoattractant Protein-1 Synthesis by Murine Lung Fibroblasts Modulates CD4+ T Cell Activation

Cory M. Hogaboam,* Nicholas W. Lukacs,* Stephen W. Chensue,*‡ Robert M. Strieter, † and Steven L. Kunkel2*

This study addressed the role of endogenous monocyte chemoattractant protein-1 (MCP-1) in Ag-stimulated lymphokine synthesis and proliferation by CD4+ T cells during their coculture with purified lung fibroblasts or splenic macrophages. Initial experiments showed that fibroblasts exposed to IL-4, TNF α, or IL-4 and TNF-α (all at 10 ng/ml) for 24 h released five- to eightfold more MCP-1 than similarly treated splenocytes. In 72-h coculture experiments, the synthesis of IL-4 by OVA-activated CD4+ T cells added to lung fibroblasts or splenic macrophages was significantly inhibited when endogenous MCP-1 was neutralized using polyclonal anti-MCP-1 antiserum. In these same cocultures, IFN-γ levels were significantly enhanced. Similarly, IFN-γ levels were significantly enhanced in 72-h cocultures of a purified peptide derivative-activated CD4+ Th1 clone and lung fibroblasts or splenic macrophages following immunoneutralization of MCP-1. In separate experiments, the selective inhibition of MCP-1 synthesis by lung fibroblasts and splenic macrophages using an MCP-1 antisense oligonucleotide significantly enhanced the proliferation of CD4+ T cells during a 96-h coculture. Taken together, these data suggest that MCP-1 exerts an immunomodulatory effect on CD4+ T cell-derived IL-4 and IFN-γ release and CD4+ T cell proliferation during cell-to-cell interactions. The Journal of Immunology, 1998, 160: 4606–4614.

Chronic inflammatory lung diseases are often associated with lung fibroblast activation due, in part, to the modulating effects of T lymphocyte-derived cytokines (1). Recent evidence suggests that the interaction between T cells and lung structural cells such as the fibroblast during inflammatory reactions is actually bidirectional (2). A complete understanding of the reciprocal communication between immune and nonimmune cells has been elusive, but it is postulated that complimentary cell surface elements or soluble mediators dictate the nature and the extent of this cell-to-cell interaction. Chemotactic cytokines or chemokines have been shown to contribute to inflammatory reactions in the lung (3), and therefore may participate in the mediation of bidirectional immune cell-to-nonimmune cell interactions. Various inflammatory events promote the synthesis and release of chemokines, which are structurally divided into a number of families based on the arrangement of their N-terminal cysteines (4). The best described chemokine families are the C-C and C-X-C chemokines. C-C chemokines include macrophage chemotactic protein-1α (MCP-1α),3 eotaxin, C10, and monocyte chemoattractant protein-1 (MCP-1) (5).

MCP-1 was first identified as a platelet-derived growth factor-inducible product (JE) from murine 3T3 fibroblasts (6, 7). Since this initial discovery, MCP-1 synthesis has been detected in synoviocytes, endothelium (8), epithelial cells (9, 10), fibroblasts (8, 11), monocytes/macrophages, vascular smooth muscle cells (12, 13), mesangial cells, and keratinocytes (14). MCP-1 has been implicated in various diseases in which infiltrating monocytes and/or lymphocytes exert a prominent effect during the inflammatory process (15), including rheumatoid arthritis (16), inflammatory bowel disease (17), and idiopathic pulmonary fibrosis (18, 19). In experimental models of disease, numerous studies have illustrated the contrasting pathogenic and protective role of MCP-1. For example, MCP-1 contributes to delayed-type hypersensitivity responses in rats (20), the inflammatory cell infiltration into the lungs of bleomycin-treated mice (21), and mediates vessel aortic smooth muscle injury (22). However, MCP-1 is necessary for the containment of Cryptococcus neoformans through its effect on the recruitment of CD4+ T cell and monocyte/macrophage into the lungs of infected mice (23). Thus, the regulated synthesis of MCP-1 is critical to the initiation and maintenance of inflammatory responses in various organs and tissues.

While chemokines clearly mediate leukocyte infiltration into injured or inflamed tissue sites, recent findings also suggest that chemokines may have additional immunoregulatory roles (3). For example, the systemic overexpression of MCP-1 in transgenic mice demonstrated no overt monocyte accumulation in organs, but these mice were susceptible to infection by intracellular parasites (24). When MCP-1 was selectively overexpressed in the murine lung,

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3Abbreviations used in this paper: MIP-1α, macrophage inflammatory protein-1α; CCR2, chemokine receptor-2; MCP-1, monocyte chemoattractant protein-1; PPD, purified peptide derivative; RPMI-10, RPMI-1640 medium containing 10% fetal bovine serum; SEA, Schistosoma mansoni egg antigen.

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monocyte recruitment to the lung was evident, yet lung injury was only observed when an inflammatory stimulus was delivered (25). Separate studies have shown that MCP-1 contributes to the evolution of Th2 pulmonary granulomas, possibly through MCP-1 modulation of lymphocyte IL-4 production (26). Attenuation of experimental autoimmune encephalomyelitis was correlated with increased MCP-1 synthesis and decreased MIP-1α. In addition, exogenous MCP-1 attenuated the transfer of disease in this model (27). These studies support the hypothesis that MCP-1 may play an important regulatory role in these and other inflammatory or immune responses via novel effects on lymphocyte function.

The present study addressed the role of MCP-1 in the regulation of CD4+ T cell activation during coculture with normal lung fibroblasts or splenic macrophages. We examined the effects of MCP-1 released by lung fibroblasts and splenic macrophages on two aspects of T cell activation, namely de novo IL-4 and IFN-γ generation and T cell proliferation. These responses were tested using purified splenic CD4+ T cells from mice sensitized to OVA in the presence of either CFA-OVA or Schistosoma mansoni egg Ag (SEA-OVA). In these experiments, isolated CD4+ T cells from both Ag sensitization models were mixed in equal proportions to obtain a CD4+ T cell population that generated IL-4 and IFN-γ following OVA rechallenge. A 72-h coculture of CD4+ T cells with lung fibroblasts or splenic macrophages demonstrated that endogenous MCP-1 was necessary for IL-4 generation by the OVA-stimulated CD4+ T cells. In the absence of MCP-1, IFN-γ levels in these same cocultures were significantly increased. Further, immunoneutralization of MCP-1 during a 72-h coculture of a murine CD4+ Th1 clone reactive to purified-peptide derivative (PPD) from Mycobacterium with lung fibroblasts or splenic macrophages significantly enhanced IFN-γ levels. To address the effect of MCP-1 on CD4+ T cell proliferation, MCP-1 synthesis by lung fibroblasts and splenic macrophages was inhibited by using an MCP-1 antisense oligonucleotide during a 96-h coculture of these cells and CD4+ T cells. In these experiments, OVA-specific proliferative responses by CD4+ T cells were significantly enhanced, not only during coculture with splenic macrophages, but also during coculture with lung fibroblasts. These data show a dichotomous influence of MCP-1 on IL-4 and IFN-γ synthesis by CD4+ T cells, and a modulatory effect on the proliferation of CD4+ T cells during cell-to-cell interactions.

Materials and Methods

Animals
Female CBA/J mice (5–7 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed under specific-pathogen-free conditions with access to food and water at all times. All procedures described herein were approved by the University Laboratory Animal Care Committee of the University of Michigan Medical School. Before purification of OVA-sensitized CD4+ splenic T cells (see below), mice were injected i.p. with 400 μg of chicken egg albumin, fraction V (OVA; Sigma, St. Louis, MO) in combination with either 0.5 ml of CFA diluted 1:1 with normal saline (CFA-OVA) or 3000 freshly isolated S. mansoni eggs suspended in 0.5 ml of normal saline (SEA-OVA).

Purification of splenic CD4+ T lymphocytes from OVA-sensitized mice
Fourteen days after sensitization, spleens were aseptically removed from either CFA-OVA or SEA-OVA mice and suspended in RPMI containing 10% FBS (RPMI-10). BRC were lysed by the cell suspensions using a hypotonic lysing buffer (150 mM NH₄Cl; 10 mM NaHCO₃; 1 mM EDTA) for 2 min at 4°C and the remaining cells were added to six-well tissue culture plates. After incubating for 2 h at 37°C, the supernatants containing nonadherent splenocytes were removed, and these cells were further purified to yield cell preparations of CD4+ T cells. Briefly, the nonadherent spleen cells were treated with anti-mouse Ly2 (CD8; YTS 169.4; Accurate, Westbury, NY) for 1 h at 4°C. CD4+ T cells were then recovered by eluting the Ab-treated spleen cells through a Cedarlane Mouse CD4 Recovery Column (Accurate) with 20 ml of RPMI-10 (37°C). According to flow cytometric analysis, ~85% of the cells eluted from the CD4 recovery column were CD4+ T cells. No CD8+, CD23+, or ED1+ cells were detected in cell suspensions of eluted cells.

Initial studies showed that CD4+ T cells purified from CFA-OVA and SEA-OVA mice had polarized IFN-γ and IL-4, respectively, following OVA rechallenge during a 72-h coculture with lung fibroblasts or splenic macrophages. To obtain a CD4+ T cell population from which IL-4 and IFN-γ production was consistently demonstrable, CFA-OVA and SEA-OVA CD4+ T cells were mixed in equal proportions before coculture experiments (see below). Mixed CFA-OVA and SEA-OVA CD4+ T cells produced both IL-4 and IFN-γ following OVA rechallenge in vitro.

Normal lung fibroblast cell cultures
 Cultures of normal lung were isolated and cultured as previously described in detail (11). Fibroblasts were grown at 37°C in a humidified CO2 incubator and fed DMEM containing 1% (v/v) antibiotic-antimycotic and 15% (v/v) FBS. These cells were expanded in 175-ml tissue culture flasks, and after a minimum of two passages, homogenous populations of fibroblasts were transferred to 6-well or 96-well tissue culture plates for experiments. Cultured lung fibroblasts were used in these experiments up to the sixth passage. For immunocytochemical characterization, fibroblasts were transferred to 2-well Lab-Tek (Nunc, Naperville, IL) chamber culture slides. Cells stained for α-actin and desmin suggested a myofibroblast-type phenotype, and these cultures were found to be completely free of α-naphthyl acetate esterase-positive macrophages (data not shown). Five days before each experiment, each well in a 6-well and 96-well tissue culture plate was initially seeded with ~1 × 10⁵ and 1 × 10⁶ fibroblasts, respectively.

Splenic macrophage cultures
Splenoocytes were aseptically removed from nonsensitized mice, dissociated, and suspended in RPMI-10. BRC were lysed by the cell suspensions using a hypotonic lysing buffer for 2 min at 4°C and the remaining cells were added to six-well tissue culture plates. After incubating at 37°C for 2 h, the supernatants containing nonadherent splenocytes were removed. The splenic macrophages were maintained in DMEM growth medium for 5 days before the use of these cells in an experiment. Each well of a six-well tissue culture plate contained approximately 1 × 10⁶ adherent cells, of which approximately 95% were α-naphthyl acetate esterase-positive macrophages. α-Naphthyl acetate esterase staining was performed according to a protocol supplied by Sigma.

Lymphokine generation by purified CD4+ T cells or Th1 clones following a 72-h coculture with lung fibroblasts or splenic macrophages
The experimental protocol employing cocultures of purified OVA-reactive CD4+ T cell and isolated lung fibroblasts or splenic macrophages is summarized in Figure 1. Lung fibroblasts and splenic macrophages in six-well tissue culture plates were left untreated, or exposed to murine IL-4 (R&D Systems, Minneapolis, MN), human TNF-α (Genzyme, Cambridge, MA), or IL-4 + TNF-α (all cytokines at 10 ng/ml) in RPMI-10 containing 200 μg/ml of OVA for 24 h (step 1). IL-4 and TNF-α have previously been shown to be potent inducers of MCP-1 synthesis by endothelial cells (28), lung fibroblasts (11), and lung epithelial cells (9). Following a 24-h pre-treatment of the lung fibroblasts or splenic macrophages, a 500-μl aliquot was removed and stored at −20°C for measurement of MCP-1 levels. These cells were then thoroughly washed with buffered HBSS to remove all exogenous cytokine and OVA (step 2), and 1 × 10⁶ purified CD4+ T cells in 1 ml of RPMI-10 containing 200 μg of OVA were added to the cocultures (step 3). An equivalent mixture (i.e., 5 × 10⁶ of each) of CFA-OVA and SEA-OVA CD4+ T cells was used in these experiments. Equivalent numbers of CD4+ T cells were also added to wells lacking lung fibroblasts or splenic macrophages. To address the role of MCP-1 on T cell lymphokine generation during the coculture, normal rabbit serum or rabbit anti-MCP-1-neutralizing polyclonal Ab (11) was added at the beginning of the coculture period (step 3). After 72 h in coculture, a cell-free supernatant was removed from each well (step 4) for chemokine and cytokine measurements (see below).

In separate experiments, lung fibroblasts or splenic macrophages cultured in 96-well plates were treated with IL-4, IFN-γ, TNF-α, IL-4 + TNF-α, or IFN-γ + TNF-α (all at 10 ng/ml) for 24 h. Following the pretreatment, these cells were washed thoroughly, and 1 × 10⁶ PPD-reactive Th1 cloned cells (kindly provided by Dr. Dennis D. Taub, National Institute of Aging, Gerontology Research Center, Baltimore, MD) were added to the
lung fibroblasts or splenic macrophages. Either normal rabbit serum or anti-MCP-1-neutralizing Ab was also added. Seventy-two hours later, cell-free supernatants were removed for the measurement of IL-4 and IFN-γ by ELISA.

Effect of endogenous MCP-1 on the proliferation of purified CD4⁺ T cells following a 96-h coculture with lung fibroblasts or splenic macrophages

In experiments designed to address the role of MCP-1 in CD4⁺ T cell proliferation, MCP-1 synthesis was inhibited using purified phosphorothioated MCP-1 antisense oligonucleotide (5'-AAG CGT GAC AGA GAC CTG CAT AGT GGT GG-3'; 10 nM final concentration), which was added during the pretreatment and coculture period. Purified phosphorothioated MCP-1 sense oligonucleotide (5'-CCA CCA TCA TGC AGG TCT CTG TCA CGC TT-3'; 10 nM final concentration) was added to other control cultures at the same time points. To assess CD4⁺ T cell proliferation, these cells were cocultured with fibroblasts or splenic macrophages in 6-well tissue culture plates for 96 h. Equivalent numbers of CD4⁺ T cells were also added to wells lacking lung fibroblasts or splenic macrophages for the same time period. Changes in proliferation by the Th1 clones were not examined due to a high background of [³H]TdR incorporation by these cells. After 96 h, the CD4⁺ T cells were transferred to 96-well tissue culture plates (Fig. 1, step 5) and incubated at 37°C for 24 h in the presence of 1 µCi of [³H]TdR (ICN Radiochemicals, Irvine, CA) and 200 µg of OVA (Fig. 1, step 6). Fibroblast or splenic macrophage coculture of the transferred CD4⁺ T cells was consistently below 1%. Finally, the CD4⁺ T cells were suctioned from each well of the 96-well tissue culture plates onto glass fiber filter strips (Cambridge Technology, Watertown, MA) using a PhD Cell Harvester (Cambridge Technology). Sections on the filter strips corresponding to each well of the tissue culture plate were punched out, and the pieces were transferred to scintillation vials. [³H]TdR incorporation by CD4⁺ T cells was then determined by liquid scintillation counting on a Beckman counter (Beckman Instruments, Fullerton, CA).

Chemokine and cytokine measurement

Murine MCP-1, IL-4, and IFN-γ levels were determined in 50-µl supernatant samples using a standardized sandwich ELISA as previously described (19). Briefly, Nunc-immuno ELISA plates (MaxiSorp; Naperville, IL) were coated with the appropriate cytokine capture Ab at a dilution of 1 µg/ml of coating buffer (0.6 M NaCl, 0.26 M H₃BO₃, 0.08 M NaOH, pH 9.6) for 16 h at 4°C. The excess amount of capture Abs was washed away and each plate was blocked for 90 min with 2% BSA-PBS at 37°C. After the blocking period, each ELISA plate was washed with PBS Tween-20 (0.05%; v/v), and samples (no dilution or 1:10; 50-µl vol) were added to wells in duplicate for 1 h at 37°C. Recombinant murine MCP-1, IL-4, and IFN-γ standard curves were used to calculate chemokine or cytokine concentrations. The plates were then thoroughly washed and the appropriate biotinylated polyclonal rabbit anti-cytokine Ab (3.5 µg/ml) was added (11). After washing the plates 30 min later, streptavidin-peroxidase (Bio-Rad, Richmond, CA) was added to each well for 30 min, and each plate was thoroughly washed again. Chromagen substrate (Bio-Rad) was added and plates were read on an ELISA plate scanner at 492 nm. The limit of detection for each cytokine and chemokine was consistently above 10 pg/ml.

Statistical analysis

Purified CD4⁺ T lymphocytes were used from a total of four OVA-sensitized mice (CFA-OVA and SEA-OVA), and all test conditions were completed in duplicate for cytokine and chemokine measurements and in triplicate for a proliferation assay. Results are expressed as mean ± SE of the mean of a minimum of three separate experiments. ANOVA and the Newman-Keuls multiple comparison test were used to determine statistical significance between control and experimental groups; p < 0.05 was considered statistically significant.

Results

Differential MCP-1 synthesis by lung fibroblasts and splenic macrophages

De novo synthesis of MCP-1 by murine lung fibroblasts and splenic macrophages is shown in Figure 2. After 24 h, 22 ± 6 ng/ml of MCP-1 was detected in cultures of untreated lung fibroblasts whereas approximately four- to sixfold more MCP-1 was measured in fibroblast cultures treated with either IL-4, TNF, or both cytokines. No differences in MCP-1 levels were observed between the cytokine treatment groups. When compared with untreated fibroblasts, untreated splenic macrophages constitutively produced markedly less MCP-1 (8 ± 1 ng/ml) over the same time period. Further, MCP-1 synthesis by cytokine-treated splenic macrophages was not increased after 24 h.

MCP-1 modulates IL-4 production by purified CD4⁺ T cells during coculture with lung fibroblasts or splenic macrophages

In the investigations designed to assess the effect of MCP-1 released from lung fibroblasts or splenic macrophages on CD4⁺ T cell lymphokine synthesis, we first assessed the IL-4 levels in 72-h cocultures containing or lacking anti-MCP-1-neutralizing Ab. When cellfree supernatants were examined, immunoreactive MCP-1 was not detected in any of the cell cocultures treated with...
the anti-MCP-1-neutralizing Ab (data not shown). In these experiments an equivalent number of isolated CD4⁺ T cells from CFA-OVA- and SEA-OVA-sensitized mice was cocultured with lung fibroblasts or splenic macrophages. These CD4⁺ T cell populations were mixed to simulate an equal proportion of Th cytokine populations of CD4⁺ T cells, since OVA rechallenge of pure populations of CFA-OVA or SEA-OVA CD4⁺ T cells cocultured with lung fibroblasts for 72 h polarized lymphokine synthesis toward IFN-γ and IL-4, respectively (Table I).

The mixed CD4⁺ T cells released less than 0.05 ng/ml of IL-4 following OVA rechallenge when cultured alone; however, when cultured with fibroblasts or splenic macrophages, these mixed CD4⁺ T cells produced levels of IL-4 that exceeded 3 ng/ml. The IL-4 detected in these cocultures was the result of endogenous production of IL-4 by the CD4⁺ T cells, since exogenous IL-4 was not detected in cytokine-treated cultures after washing (Fig. 1, step 2). Clear differences in IL-4 levels in cocultures of CD4⁺ T cells and lung fibroblasts or splenic macrophages were apparent between the control serum and anti-MCP-1 Ab treatment groups at the 72-h time point (Fig. 3). The nature of the cytokine treatment of the lung fibroblasts or splenic macrophages exerted a direct effect on subsequent IL-4 generation during the coculture period because the greatest levels of IL-4 were detected in cocultures of CD4⁺ T cells and lung fibroblasts (Fig. 3A) or splenic macrophages (Fig. 3B) treated with IL-4 and TNF-α. Levels of IL-4 production by CD4⁺ T cells cocultured with fibroblasts were similar to those generated by these T cells during coculture with splenic macrophages (Fig. 3, A vs B). However, significant reductions in IL-4 due to the presence of anti-MCP-1 Ab were observed in cocultures of CD4⁺ T cells and lung fibroblasts or splenic macrophages (Fig. 3B).

**Immunoneutralization of MCP-1 during CD4⁺ T cell coculture with either lung fibroblasts or splenic macrophages enhances CD4⁺ T cell IFN-γ production**

It was next determined whether changes in IL-4 following immunoneutralization of MCP-1 were associated with alterations to CD4⁺ T cell-derived IFN-γ. Significantly higher levels of IFN-γ were apparent when anti-MCP-1-neutralizing Ab was included in cocultures of CD4⁺ T cells and cytokine-treated lung fibroblasts (Fig. 4A). In addition, significant elevations in IFN-γ levels were present in all cocultures of mixed CFA-OVA and SEA-OVA CD4⁺ T cells and splenic macrophages when MCP-1 was immunoneutralized (Fig. 4B). Specifically, fourfold increases in IFN-γ were observed in cocultures of T cells and IL-4-treated splenic macrophages. These data suggested that both IL-4 and IFN-γ levels were affected by endogenous MCP-1 production during T cell coculture with either fibroblasts or splenic macrophages.

**Immunoneutralization of MCP-1 increases IFN-γ synthesis by Th1 clones cocultured with lung fibroblasts or splenic macrophages**

From the previous set of experiments, alterations in IFN-γ synthesis by mixed CFA-OVA and SEA-OVA CD4⁺ T cells appeared to be enhanced by the neutralization of MCP-1. To address this observation more fully, we assessed whether MCP-1 had any direct effect on the ability of a PPD-reactive Th1 clone to produce IFN-γ following Ag rechallenge. When cultured in the absence of fibroblasts or splenic macrophages, the Th1 clone did not produce detectable quantities of IL-4 or IFN-γ following PPD rechallenge for 72 h (Table II). In addition, no IL-4 was detected during PPD-rechallenge of the Th1 cell clone during a 72-h coculture with untreated or cytokine-treated lung fibroblasts or splenic macrophages. IFN-γ was detected in all cocultures of Th1 cells and cytokine-treated lung fibroblasts except in cocultures of TNF-α-treated fibroblasts and Th1 cells. In the presence of anti-MCP-1 Ab, Th1-derived IFN-γ was significantly enhanced in all cocultures after 72 h (Table II).

**Table I. IL-4 and IFN-γ generation by purified, splenic CD4⁺ T cells from CFA- and OVA-sensitized mice and SEA-sensitized mice**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Generation of Cytokine (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFA-OVA CD4⁺ T cells</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.40 ± 0.82</td>
</tr>
</tbody>
</table>

* CFA-OVA or SEA-OVA CD4⁺ T cells were cocultured with lung fibroblasts or splenic macrophages in the presence of OVA for 72 h. Results are mean ± SE from three separate experiments.
cytokine-treated splenic macrophages, IFN-γ levels varied between 0.17 and 1.7 ng/ml. The differences in IFN-γ generation during the 72-h coculture appeared to be dependent on the nature of the splenic macrophage pretreatment (Table II). Immunoneutralization of MCP-1 significantly increased IFN-γ synthesis by Th1 cells approximately 2-fold when these cells were cocultured with IL-4- or IFN-γ-treated splenic macrophages. IFN-γ levels were decreased in cocultures of Th1 cells and TNF-α-treated splenic macrophages, but levels of IFN-γ were increased 10-fold in cocultures of Th1 cells and IL-4 + TNF-α-treated splenic macrophages. Similarly, IFN-γ levels in cocultures of Th1 cells and IFN-γ + TNF-α-treated splenic macrophages were significantly enhanced at 72 h when MCP-1 was immunoneutralized. Thus, these data suggested that MCP-1 modulated the production of IFN-γ by Th1 clones during coculture with lung fibroblasts or splenic macrophages.

**Inhibition of endogenous MCP-1 synthesis significantly augments CD4+ T cell proliferation during coculture with lung fibroblasts or splenic macrophages**

The proliferative response of CD4+ T cells during their coculture with fibroblasts or splenic macrophages was next examined. Although lung fibroblasts are not normally considered professional APCs, it has been shown that lung fibroblasts express MHC II and present Ag to T cells (29). Murine fibroblasts have also been shown to express the costimulatory molecule CD80 (B7-1), which

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**FIGURE 3.** IL-4 levels in cocultures of lung fibroblasts (A) or splenic macrophages (B) and CD4+ T cells in the presence of normal rabbit serum or anti-MCP-1-neutralizing Ab. Lung fibroblasts and splenic macrophages were treated for 24 h in the absence or presence of IL-4, TNF-α, or IL-4 + TNF-α (all at 10 ng/ml); thoroughly washed; and 5 × 10^5 CD4+ T cells purified from CFA-OVA mice combined with 5 × 10^5 purified CD4+ T cells from SEA-OVA mice were added. IL-4 levels shown here represent endogenous synthesis by added CD4+ T cells since no exogenous IL-4 was detected in the cultures of adherent cells after washing. IL-4 was measured using a specific ELISA after 72 h of coculture as described in Materials and Methods, and data represent mean ± SE from three separate experiments. *, p ≤ 0.05 compared with control fibroblasts or splenic macrophages in the normal serum treatment group.

**FIGURE 4.** IFN-γ levels in cocultures of lung fibroblasts (A) or splenic macrophages (B) and CD4+ T cells in the presence of normal rabbit serum or anti-MCP-1-neutralizing Ab. Lung fibroblasts and splenic macrophages were treated for 24 h in the absence or presence of IL-4, TNF-α, or IL-4 + TNF-α (all at 10 ng/ml); thoroughly washed; and 5 × 10^5 CD4+ T cells purified from CFA-OVA mice combined with 5 × 10^5 purified CD4+ T cells from SEA-OVA mice were added. IFN-γ levels were measured using a specific ELISA after 72 h of coculture as described in Materials and Methods, and data represent mean ± SE from two separate experiments. *, p ≤ 0.05 compared with similar cocultures in the normal serum treatment group.
Table II. Effect of anti-MCP-1 Ab on IL-4 and IFN-γ generation by PPD-reactive Th1 clones during a 72-h coculture with untreated or cytokine-treated lung fibroblasts or splenic macrophages

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pretreatment*</th>
<th>IFN (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. Pre-immune Ab</td>
<td>B. Anti-MCP-1 Ab</td>
</tr>
<tr>
<td>Th1 clone alone</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TH1 cells + lung fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.2 ± 0.01</td>
<td>0.8 ± 0.04*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.5 ± 0.04</td>
<td>0.9 ± 0.07*</td>
</tr>
<tr>
<td>TNF</td>
<td>ND</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>IL-4 + TNF</td>
<td>0.4 ± 0.03</td>
<td>0.5 ± 0.01*</td>
</tr>
<tr>
<td>IFN-γ + TNF</td>
<td>0.3 ± 0.03</td>
<td>0.5 ± 0.01*</td>
</tr>
<tr>
<td>Th1 cells + splenic macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.7 ± 0.1</td>
<td>3.1 ± 0.06*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.6 ± 0.05</td>
<td>3.6 ± 0.1*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.2 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4 + TNF-α</td>
<td>0.17 ± 0.01</td>
<td>1.1 ± 0.02*</td>
</tr>
<tr>
<td>IFN-γ + TNF-α</td>
<td>1.0 ± 0.02</td>
<td>1.5 ± 0.05*</td>
</tr>
</tbody>
</table>

* Lung fibroblasts or splenic macrophages in 96-well tissue culture plates were treated with IL-4, IFN-γ, TNF-α, IL-4 + TNF-α or IFN-γ + TNF-α (all at 10 ng/ml) for 24 h. Following the pretreatment, these cells were washed thoroughly, and cell-free supernatants were removed for the measurement of IFN-γ. IFN-γ and IFN-γ by ELISA. No IL-4 was detected in the cell supernatants under the conditions examined.

Discussion

The ability of MCP-1 to alter CD4+ T cell activation represents a potentially novel immunoregulatory mechanism for chemokines. Previous studies have shown that C-C chemokines can direct the migration of T cells (31–33) and that T cells appear to express a rather limited repertoire of chemokine receptors under specific conditions. Expression of chemokine receptor-2 (CCR2) is limited to IL-2-activated T cells (34), and MCP-1 binding to CCR2 induces the chemotaxis of these cell types (31, 35, 36). The observation that activated T cells express CCR2 supports the hypothesis that MCP-1 plays an important role in regulating the activation of these cells. From the present study, we ascertained that MCP-1 synthesis by untreated or cytokine-treated fibroblasts and splenic macrophages plays a prominent role in the regulation of CD4+ T

Table III. Effect of sense and antisense MCP-1 oligonucleotides on MCP-1 generation in 96-h cocultures of lung fibroblasts or splenic macrophages and CD4+ T cells

<table>
<thead>
<tr>
<th>Cells: Cytokine pretreatment*</th>
<th>MCP-1 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+MCP-1 sense oligonucleotide (20 nM)</td>
</tr>
<tr>
<td>Lung fibroblasts</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>IL-4</td>
<td>123 ± 3</td>
</tr>
<tr>
<td>TNF</td>
<td>108 ± 10</td>
</tr>
<tr>
<td>IL-4 + TNF</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>Splenic macrophages</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>IL-4</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>TNF</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>IL-4 + TNF</td>
<td>26 ± 5</td>
</tr>
</tbody>
</table>

* Oligonucleotides were added at the beginning of the pretreatment and coculture periods (see Fig. 1).

* Lung fibroblasts or splenic macrophages in 96-well tissue culture plates were treated with IL-4, TNF-α, or IL-4 + TNF-α (all at 10 ng/ml) for 24 h in the presence of MCP-1 sense oligonucleotide or MCP-1 antisense oligonucleotide (both at 10 nM). Following the pretreatment period, the cells were washed thoroughly, and 1.0 × 10^6 splenic CD4+ T cells were added with either MCP-1 sense oligonucleotide or MCP-1 antisense oligonucleotide (both at 10 nM). Ninety-six hours later, cell-free supernatants were removed for the measurement of MCP-1 by ELISA.

* ND = none detected.
cell cytokine production and proliferation during cell-to-cell interactions. The immunoneutralization of MCP-1 in cultures of lung fibroblasts or splenic macrophages negatively affected IL-4 synthesis by purified CD4+ T cells following OVA rechallenge. The effect of anti-MCP-1 Ab on IL-4 generation by OVA-rechallenged CD4+ T cells was observed when equivalent mixtures of CD4+ T cells from CFA-OVA and SEA-OVA-sensitized mice were used in the coculture experiments. Interestingly, normal lung fibroblasts were equally effective as splenic macrophages in the promotion of IL-4 synthesis by CD4+ T cells. Further, in the absence of MCP-1, IFN-γ synthesis by CD4+ T cells was augmented, most notably during their coculture with splenic macrophages. This finding was consistent with the increased IFN-γ levels measured in cocultures of PPD-reactive Th1 cells and IL-4-treated fibroblasts or splenic macrophages. Inhibition of MCP-1 synthesis by lung fibroblasts and splenic macrophages by ≈46% using an MCP-1 antisense oligonucleotide enhanced the proliferation of mixed CD4+ T cells during their coculture with either adherent cell type. These data suggest that MCP-1 production by stromal cells such as lung fibroblasts could be important in the generation of Th2-type responses characterized by IL-4 synthesis (37, 38), and it may be one regulating factor that prevents inappropriate T cell activation in the lung, spleen, and other organs.

The present findings build on our previous observations that MCP-1 exerts a dichotomous effect on Th cell cytokine synthesis in the inflamed lung and in the spleen. Recent investigations have shown that MCP-1 contributes to experimental granulomatous inflammation in the lung dictated by Th2-type cells. From those studies, levels of MCP-1 were higher in the Th2 granuloma than in the Th1 type, and depletion of MCP-1 reduced the size of the Th2 lesion without affecting the size of the Th1-type lesion. In other studies, we showed that MCP-1 directly contributed to IL-4 synthesis in mixed spleen cell cultures (26). Karpus et al. (39) have confirmed these observations by showing that the presence of MCP-1 during OVA-specific TCR-transgenic T cell activation enhances IL-4 production. It is well appreciated that fibroblasts express receptors for IL-4 and respond to IL-4 by increasing extracellular matrix protein production (40, 41). In contrast to the potentiating effects of MCP-1 on IL-4 synthesis by CD4+ T cells, the reduction of MCP-1 levels was shown to enhance IFN-γ generation by these T cells and a Th1 clone during their coculture with lung fibroblasts or splenic macrophages. Studies are currently being performed to examine the exact regulatory mechanism through which MCP-1 regulates IL-4 and IFN-γ generation by T cells. This mechanism conceivably occurs at the gene transcription level, although posttranslational mechanisms may also be operations since the Th1 clone could not be “redirected” toward a Th2 phenotype or IL-4 production by the presence of endogenous MCP-1. The same was shown with the addition of exogenous MCP-1 to cocultures of the Th1 clone and splenic macrophages where IFN-γ levels were decreased, but IL-4 was not detected (our unpublished observations). Thus, the present data suggest that MCP-1 has a direct role in T cell activation and may be particularly important in the modulation of Th2-type responses through a currently undefined mechanism.

Enhanced IFN-γ synthesis by CD4+ T cells is potentially deleterious to tissues such as the lung, since it has been shown that fibroblasts may perpetuate CD4+ T cell activation following exposure to IFN-γ through the expression of MHC II and CD80 (B7-1) (29, 30). The present findings concur with these previous observations in that in the absence of endogenous MCP-1 production by fibroblasts, mixed CFA-OVA and SEA-OVA CD4+ T cells had a greater propensity for proliferation after a 96-h coculture. This enhancement was also apparent in cocultures of CD4+ T cells and splenic macrophages, which are well-described professional APCs. Extensive research has shown that T cell proliferation requires a complex array of cell-to-cell signals in addition to interaction between the TCR-MHC II bound to processed Ag, but numerous adhesion and/or soluble molecules have been shown to supply a secondary or costimulatory signal (42) necessary for T cell proliferation. More recent work by Taub et al. (43) has shown that C-C chemokines such as MIP-1α, macrophage inflammatory protein-1β, RANTES, and MCP-1 are also costimulatory candidates in that these chemokines appear to enhance mitogen or Ag-mediated human T cell activation. While maximal human T cell proliferation in these studies occurred at chemokine concentrations below 10 ng/ml (43), we observed inhibitory effects on murine T cell proliferation when MCP-1 concentrations exceeded 20 ng/ml.

FIGURE 5. Proliferation of mixed CFA-OVA and SEA-OVA CD4+ T cells cultured alone or following a 96-h coculture with lung fibroblasts or splenic macrophages in the presence of MCP-1 sense oligonucleotide or MCP-1 antisense oligonucleotide. In these experiments, 5 × 10^6 CD4+ T cells purified from CFA-OVA mice combined with 5 × 10^6 purified CD4+ T cells from SEA-OVA mice were added to untreated or cytokine-treated fibroblasts or splenic macrophages for 96 h as described in Materials and Methods. [3H]Thymidine incorporation by CD4+ T cells was monitored for 24 h following the removal of these cells from the fibroblasts or splenic macrophages. Data are mean ± SE of triplicate measurements from two separate proliferation assays.
Our data coincide with a previous report by Zhou et al. (44), who demonstrated that pretreatment of murine T cells with MIP-1α inhibited IL-2 transcription and translation typically induced by an anti-CD3 mAb stimulus. Thus, T cell activation leading to proliferation is affected by chemokines, and this effect is probably dependent not only on the concentration of the chemokine but the presence or absence of other costimulatory signals.

Structural-type cells in the lung and elsewhere have been shown to be a source of MCP-1 (8, 9, 11, 28). Data from this study and previous studies support the concept that all of the MCP-1 synthesis observed in these cocultures was attributable to fibroblasts or splenic macrophages, since CD4+ T cells cultured alone did not release detectable (i.e., amounts less than 50 pg/ml) quantities of MCP-1. In addition, it was apparent that lung fibroblasts produce more MCP-1 constitutively than splenic macrophages, and are responsive to cytokines such as IL-4 and TNF-α. This may be explained by the fact that the spleen plays a far more important role in immune activation than the lung, and as a consequence MCP-1 production in the spleen may compromise this function. Conversely, T cell activation in the lung could have many deleterious consequences. Accordingly, the synthesis of MCP-1 by lung fibroblasts may exert an important immunomodulatory role on the T cell and, for example, prevent excess IFN-γ production. However, it should be noted that augmented IL-4 may also have negative effects on the architecture of the lung since this cytokine can activate fibroblasts to produce collagen (41). Fibroblast interaction with T cells is not a passive event, but this interaction is conceivably balanced by a number of regulatory mechanisms that prevent the excessive activation of either cell type. MCP-1 appears to be a soluble factor produced by structural cells such as fibroblasts that participates in the regulatory balance of T cell activation.

In conclusion, the present study documents the regulatory role of MCP-1 on T cell activation during cell-to-cell interactions with normal lung fibroblasts or splenic macrophages. Taken together, these findings suggest that endogenous MCP-1 supplies an important immunomodulatory signal to immune cells such as CD4+ T cells. The modulatory effect of MCP-1 may also dictate the cytokine profile associated with a Th response. These data are impetus to further explore the role that other C-C chemokines play in regulating immune cell/nonimmune cell interactions within various tissues.

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