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Pivotal Role of CCR1-Positive Leukocytes in Bleomycin-Induced Lung Fibrosis in Mice

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We have investigated the involvement of chemokine receptor CCR1-positive cells in bleomycin-induced lung injury, a model of pulmonary fibrosis. After bleomycin challenge in C57BL/6J mice, the expression of CCR1 mRNA increased and peaked at day 7, which paralleled to the expression of its ligands, macrophage-inflammatory protein-1α and RANTES. Immunohistochemical study showed that CCR1-positive cells accumulated in the interstitial inflammatory site. Furthermore, the treatment of anti-CCR1 Ab significantly reduced the accumulation of inflammatory cells and collagen deposition, resulting in dramatic improvement of survival. These results suggest that CCR1-positive cells play significant roles in the pathogenesis of pulmonary fibrosis subsequent to bleomycin-induced lung injury, and that CCR1 could be a novel molecular target for intervention therapy against pulmonary fibrosis. The Journal of Immunology, 2000, 164: 2745–2751.

Bleomycin-induced lung injury is a model of pulmonary fibrosis. In response to bleomycin, mice develop acute alveolitis and interstitial inflammation, characterized by the sequential recruitment of neutrophils, lymphocytes, and macrophages in the first week. Subsequent to these inflammatory responses, fibrotic responses characterized by the increase of fibroblast proliferation and extracellular matrix synthesis occur in the second week. To reveal the mechanisms underlying these events, several cytokines and chemokines have been investigated in terms of their contribution to the pathogenesis of lung fibrosis. Among them TNF-α plays a key role in this model (2, 3). Neutralization of TNF-α with anti-TNF-α Ab or soluble TNF-α receptors diminished the development of lung fibrosis in mice (4, 5). TGF-β mainly participates in fibrotic responses subsequent to inflammation (6, 7), i.e., neutralization of TGF-β with anti-TGF-β Ab reduced lung fibrosis in a murine model (6).

Chemokines, including macrophage-inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), RANTES, MCP-2, and IFN-inducible protein-10 (IP-10) might also contribute to the inflammatory, fibrotic, and angiogenic responses in a bleomycin model (8–10). Furthermore, analyses of bronchoalveolar lavage (BAL) and open-lung biopsies from patients with idiopathic pulmonary fibrosis have demonstrated elevated levels of MIP-1α, RANTES, and MCP-1 compared with that of healthy volunteers (11–13). A CC chemokine, MIP-1α, promotes leukocyte accumulation and activation, leading to fibrosis. Treatment of bleomycin-challenged mice with anti-MIP-1α Ab reduced accumulation of pulmonary mononuclear phagocytes and fibrosis (8). RANTES, another CC chemokine, is a potent eosinophil and lymphocyte attractant and may mediate T lymphocyte influx in fibrosing alveolitis (9, 10).

Although it is clear that macrophages, lymphocytes, neutrophils, eosinophils, and their chemoattractants participate in the pathogenesis of lung fibrosis, it is still controversial which subtype of leukocyte plays an essential role (15, 16). Since chemokines induce their biological effects by interacting with specific receptors on the target cell surface, the chemokine receptors have been expected to be the targets to prevent leukocyte migration or activation causing fibrosis.

CCR1 is constitutively expressed on monocytes, neutrophils, lymphocytes, and eosinophils (17, 18) and interacts with MIP-1α, RANTES, leukotactin-1, MCP-3, and hemofiltrate-derived CC chemokine-1. Recent studies showed that immature dendritic cells also express CCR1 and regulate interaction of dendritic cells with T cells in the process of Ag presentation (19, 20).

In mouse, MIP-1α has been implicated in multiple pathologies including pulmonary fibrosis, influenza A alveolitis, and experimental allergic encephalomyelitis; however, specific roles of CCR1 are not defined yet. In the present study, we investigated the role of CCR1-expressing cells in bleomycin-induced lung injury, including expression kinetics along with the production of its ligands and immunohistochemical localization. Furthermore, we examined the effects of neutralizing anti-CCR1 Ab on bleomycin-induced lung fibrosis in mice.

Materials and Methods

Animals and reagents

Specific pathogen-free female C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). The mice were maintained in a pathogen-free mouse facility at the Department of Molecular Preventive Medicine, University of Tokyo. Female New Zealand White rabbits were purchased from CLEA Japan (Tokyo, Japan). All experiments complied with approved animal care protocols of the University of Tokyo. Bleomycin sulfate was kindly gifted from Nipponkayaku (Tokyo, Japan).

Bleomycin administration

Bleomycin sulfate was administered to C57BL/6J female mice aged 8–10 wk. Briefly, C57BL/6J mice were anesthetized with 200 µl of 5 mg/ml

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pentobarbital injected i.p., followed by intratracheal instillation of 3 mg/kg bleomycin sulfate in 50 μl sterile saline.

Preparation of a recombinant GST protein fused with the NH2-terminal portion of murine CCR1
cDNA encoding the NH2-terminal extracellular portion of murine CCR1 was obtained by PCR using the full-length cDNA as a template and a set of oligonucleotides (5'-GCGGATCCATGGAGATTTCAGATTTCA CAG-3' and 5'-GGGCGGGCGCTTCCAAAGGCTCTACAGC-3') as primers (17). The resulting fragment was digested with BamHI and NotI and subcloned into a GST fusion protein expression vector, pGEM-T3, which was predigested with BamHI and NotI. Expression and purification of the GST fusion protein was performed as described previously (17).

Similarly, we prepared the NH2-terminal portion of murine CCR2 using the full-length cDNA as a template and a set of oligonucleotides (5'-GGG GATCCATGGAGACAATAATATGTTAC-3' and 5'-GGGCGGGCGC CTATCCAATTTGCTTCAACT-3') as primers (17).

Preparation of polyclonal Ab to a GST protein fused with the NH2-terminal portion of murine CCR1
Rabbit anti-CCR1 Ab was prepared by multiple-site immunization of New Zealand White rabbits with GST fusion protein in CFA (latron Laboratories, Tokyo, Japan). One week after the final immunization, rabbits were bled and sera were obtained and fractionated into IgG using a column packed with protein G-agarose (Pharmacia Biotech, Uppsala, Sweden). The anti-CCR1 Ab stained only CCR1 transfectants, but not CCR2, CCR7, or CXCR4 transfectants, establishing the binding specificity. Furthermore, 50% inhibition of MIP-1α-induced in vitro splenocytes chemotaxis was obtained at 10 μg/ml anti-CCR1 Ab. Similarly, we prepared blocking anti-CCR2 Ab with anti-CCR2 Ab stained only CCR2 transfectants, but not CCR1, CCR7, or CXCR4 transfectants. Sixty-six percent inhibition of MCP-1/JE-induced in vitro chemotaxis was obtained at 100 μg/ml anti-CCR2 Ab.

Passive immunization with anti-CCR1 Ab
to evaluate the effects of anti-CCR1 Ab or anti-CCR2 Ab on bleomycin-induced lung injury, we treated mice with Ab three times. Mice were injected i.v. with 500 μg anti-CCR1 Ab, anti-CCR2 Ab, or normal rabbit IgG in 200 μl PBS 1 h before intratracheal administration of bleomycin, and additional anti-CCR1 Ab, anti-CCR2 Ab, or normal rabbit IgG was injected i.p. at days 3 and 6.

Histopathology
Tissues were fixed in 10% buffered Formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined by light microscopy.

Assessment of BAL cells and lung-infiltrating leukocytes
BAL cells and lung-infiltrating leukocytes were prepared as described elsewhere with some modifications. In brief, after anesthesia, 1 ml PBS was instilled and withdrawn five times from the lung via an intratracheal cannula. The BAL fluids were collected and after RBC lysis total leukocyte counts were determined. Cell differentials were determined after cytospin centrifuge. Specimens were stained with Diff-Quik products (Baxter, Miami, FL).

to get the lung-infiltrating leukocytes, lungs were perfused with saline, dissected from the chest cavity, and then minced with scissors. Each sample was incubated for 30 min at 37°C on a rocker in 15 ml digesting buffer (10% FCS in RPMI 1640 with 1% collagenase; Wako Pure Chemical, Osaka, Japan). Next, the sample was pressed through nylon mesh and centrifuged at 2000 rpm for 20 min to remove lung parenchymal cells and RBC. The pellet was resuspended in 2.5% FCS-PBS after being rinsed. After cell counts were performed, flow cytometric immunofluorescence analyses were conducted.

Flow cytometry
Immunofluorescence analyses of peripheral blood leukocytes and lung-infiltrating leukocytes were performed with the use of an Epics Elite cell sorter (Coulter Electronics, Hialeah, FL) as described previously (21, 22).

Peripheral blood leukocytes were prepared from normal mice with RBC lysis buffer. After incubation with Fc Block (anti-CD16/32; PharMingen, San Diego, CA) for 10 min, cells were stained with PE-conjugated mAb against CD3, CD4, CD8, CD11b, CD11c, and Gr-1 (PharMingen), and also stained with 20 μg/ml of rabbit anti-CCR1 polyclonal Ab followed by staining with FITC-conjugated goat anti-rabbit IgG (Leinco Technologies, St. Louis, MO). Before analyses propidium iodide (Sigma) staining was performed to remove the dead cells.

Chemokine and chemokine receptor gene expression analysis in bleomycin-challenged lung
Total RNA was isolated from lung specimens using RNeasy B (Tel-Test, Friendswood, TX) according to manufacturer’s instructions. It was then reversely transcribed into cDNA and amplified. The PCR products of MIP-1α, RANTES, CCR1, CCR2, and CCR5 were examined by 2% agarose gel electrophoresis. After ethidium bromide staining, bands were visible only at the expected size for each mRNA product. The sense primer for CCR1 was 5'-GGTCCATGCTGCACTAGGTTG-3' and the antisense primer was 5'-GGTGAACAGGATGATGCTGG-3'. The sense primer for CCR2 was 5'-TTGTAACCTGAGTCCTACAGG-3' and the antisense primer was 5'-CAGAATGGTAATGTGAGCAGGAA-3'. The sense primer for MCP-1/JE was 5'-ATGCAGGTCCCTGTCATGC-3' and the antisense primer was 5'-GTTCAGGTAAATGTGAGCAGGA-3'. The sense primer for CXCL1 was 5'-ATGCAGGTCCCTGTCATGC-3' and the antisense primer was 5'-GTTCAGGTAAATGTGAGCAGGA-3'. The sense primer for CXCL2 was 5'-ATGCAGGTCCCTGTCATGC-3' and the antisense primer was 5'-GTTCAGGTAAATGTGAGCAGGA-3'. The sense primer for CXCL5 was 5'-ATGCAGGTCCCTGTCATGC-3' and the antisense primer was 5'-GTTCAGGTAAATGTGAGCAGGA-3'. The sense primer for CXCL12 was 5'-ATGCAGGTCCCTGTCATGC-3' and the antisense primer was 5'-GTTCAGGTAAATGTGAGCAGGA-3'.

Immunohistochemistry
The preparation of lung specimens was described as described previously (21, 22). Briefly, lung specimens were fixed in periodate-lysine-paraformaldehyde solution, washed with PBS containing sucrose, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and cut into 7-μm-thick sections with a cryostat. After inhibition of endogenous peroxidase activity, the sections were incubated with the first Ab. The Abs used were rabbit anti-CCR1 Ab, rat anti-F4/80 (BMA Biomedicals, Geneva, Switzerland), rat anti-CD4, rat anti-CD8, rat anti-Gr-1 (PharMingen), rat anti-nonlymphoid dendritic cell (NLDC)-145, and rat anti-MHC class II (BMA Biomedicals). As a negative control either a rabbit IgG or a rat IgG was used, respectively. They were treated sequentially with either HRP-conjugated goat anti-rabbit IgG (Cedarlane Laboratories, Hornby, Ontario, CA) or a HRP-conjugated goat anti-rat IgG (BioSource International, Camarillo, CA). After staining with 3,3'-diaminobenzidine (Wako Pure Chemical) or 3-amin-9-ethylcarbazole substrate kit (Vector Laboratories, Burlingame, CA), samples were counterstained with Mayer’s hematoxylin.

Collagen assay
Total lung collagen content was determined by assaying total soluble collagen using the Sircol Collagen Assay kit (Biocolor, Northern Ireland) according to the manufacturer’s instructions. Briefly, lungs were harvested at day 14 after bleomycin administration and homogenized in 10 ml 0.5 M acetic acid containing about 1 mg pepsin/10 mg tissue residue. Each sample was incubated for 24 h at 4°C with stirring. After centrifugation, 200 μl of each supernatant was assayed. One milliliter of Sircol dye reagent that binds to collagen was added to each sample and then mixed for 30 min. After centrifugation, the pellet was suspended in 1 ml of the alkali reagent included in the kit and read at 540 nm by a spectrophotometer. Collagen standard solutions were utilized to construct a standard curve. Collagens contain about 14% hydroxyproline by weight, and collagen contents obtained with this method correlate well with the hydroxyproline content according to the manufacturer’s data.

Statistical analysis
Results are expressed as means ± SD. Comparisons between the two groups were analyzed using paired Student’s t test or ANOVA. A generalized Wilcoxon test of Kaplan-Meier curves was used to evaluate the significance of survival rates. p < 0.05 was accepted as statistically significant.

Results
CCR1 expression on peripheral blood leukocytes
To determine which subpopulation of leukocytes expresses CCR1, two-color immunofluorescence analysis was performed (Fig. 1). A majority of CD11b+ or a part of Gr-1-positive cells was strongly stained with anti-CCR1 Ab, whereas CD3-, CD4-, or CD8-positive cells were weakly stained with anti-CCR1 Ab. There were a small number of CCR1 and CD11c double positive cells. These results

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demonstrated that murine peripheral blood monocytes, granulocytes, and possibly a part of dendritic precursor cells in peripheral blood express CCR1 on their surface, whereas lymphocytes expressed CCR1 at low levels. These data are consistent with the previous reports that MIP-1α exhibits chemotactic activity against a wide range of leukocytes, including immature dendritic cells (19, 20, 23). Furthermore, there were some differences of CCR1 distribution between murine leukocytes and human leukocytes. In murine peripheral blood, CCR1 is expressed on wide range of leukocytes including neutrophils (Fig. 1) (24). In contrast, human peripheral blood neutrophils lack expression of CCR1 (17).

Chemokine and chemokine receptor expression in bleomycin-induced lung injury
To determine chemokine or chemokine receptor expression, RT-PCR and quantitative PCR of whole-lung homogenate were performed (Fig. 2). Peripheral blood contamination was minimized by lung perfusion with saline. Low levels of CCR1, CCR2, and CCR5 mRNA were detected in the untreated lung. After bleomycin treatment, the expression levels were subsequently enhanced and peaked at day 7. Their ligands, MIP-1α, RANTES, and MCP-1/JE also peaked at day 7. Real-time quantitative PCR of CCR1 confirmed the above expression patterns (Fig. 2B). These results are consistent with the observation that leukocytes accumulate mainly in the first week (1).

FIGURE 1. Two-color immunofluorescence analyses on murine peripheral blood leukocytes. Leukocytes were analyzed with anti-CCR1 Ab in conjunction with anti-CD3, CD4, CD8, CD11b, CD11c, or Gr-1 Ab.

FIGURE 2. RT-PCR and quantitative PCR of chemokines and their receptors. A. Kinetics of MIP-1α, RANTES, MCP-1/JE, CCR1, CCR2, and CCR5 mRNA expression in the lung. Total RNA was isolated from whole-lung tissues of untreated or bleomycin-treated mice at days 1, 3, 7, and 14. RT-PCR cDNA products were generated and amplified using oligonucleotide primers specific to each chemokine, chemokine receptor, or a housekeeping gene GAPDH. B. Quantitative PCR of CCR1. The amount of CCR1 was normalized to the level of GAPDH at each time point. Normalized CCR1 value of untreated lung was designated as the calibrator, and final relative quantity of mRNA was expressed relative to the calibrator. PCR was performed in triplicate for each experiment.

The cellular composition of the infiltrating leukocytes in the lung
The cells infiltrating into the lung after bleomycin treatment were identified using immunohistochemical staining of frozen sections. At day 7 after bleomycin treatment, CCR1 protein was detected on large mononuclear cells present in the subepithelial and interstitial inflammatory area and alveolar macrophages (Fig. 3, a–c). In immunohistochemical staining, few number of alveolar macrophages was detected in untreated mice, which were CCR1 positive in immunofluorescence analysis (data not shown). F4/80-positive large cells, which were considered to be macrophages, were detected in the interstitial inflammatory area (Fig. 3d). The invading lymphocytes were mainly CD4-positive cells (Fig. 3e), with a few CD8-positive cells (Fig. 3f). Gr-1-positive small cells, which were considered to be granulocytes, were scattered in the interstitial inflammatory area (Fig. 3g). Dendritic cells were detected as NLDC-145-positive large cells in the upper layer of the epithelium and interstitial inflammatory site (Fig. 3h). MHC class II-positive cells were also detected in a similar area (Fig. 3i). Immunohistochemical staining of untreated lung barely detected NLDC-145- or MHC class II-positive cells (data not shown). These findings suggest that dendritic cells besides macrophages, lymphocytes, and
granulocytes may be also involved in these lines of pathogenesis. Moreover, many of CCR1-positive cells were considered to be macrophages or dendritic cells in both their morphology and distribution, confirming the results of immunofluorescence analysis of peripheral blood leukocytes (Fig. 1).

**Anti-CCR1 Ab protects the mice from bleomycin-induced lethal lung fibrosis**

To determine the involvement of CCR1-positive cells causing lung injury, neutralizing anti-CCR1 Ab was administered before and after bleomycin challenge. When the mice were treated with control Ab, they were getting thin and from day 7 they began to die with high lethality (45% at day 14) (Fig. 4A). Anti-CCR1 Ab treatment provided a significant protection against lethality (6.25% at day 14). In contrast, neutralization of CCR2, receptor for MCP-1/JE, showed no effect on survival (Fig. 4B).

To estimate the fibrotic changes in the lung, hematoxylin and eosin staining was performed at day 14 (Fig. 5). Control Ab-treated mice showed severely damaged lung, including inflammatory cell accumulation at the alveolar septa and disruption of the alveolar architecture associated with collagen deposition (Fig. 5, A-2). In contrast, after anti-CCR1 Ab treatment, those inflammatory and fibrotic changes were significantly reduced (Fig. 5, A-3). To further directly evaluate the collagen deposition, the total lung collagen content was determined (Fig. 5B). After bleomycin administration, the total lung collagen content was increased almost 7-fold at day 14 compared with that in untreated mice. Anti-CCR1Ab treatment reduced this collagen deposition by half. In contrast, anti-CCR2 Ab showed no significant effect, confirming the outcome of survival (Fig. 4B). These results suggest that CCR1-positive cells, rather than CCR2 positive cells, could play an essential role in bleomycin-induced fibrotic responses.

**Anti-CCR1 Ab reduced leukocyte accumulation**

We next evaluated the effects of anti-CCR1 Ab on leukocyte infiltration in bleomycin-induced inflammatory processes. We estimated the inflammatory cell populations of both BAL cells and lung-infiltrating leukocytes at 6 days after bleomycin challenge (Fig. 6). The total number of BAL cells in anti-CCR1 Ab-treated
mice was significantly decreased (by 39%) compared with that of control Ab-treated mice. Differential counts of lavage cells from anti-CCR1 Ab-treated mice showed a significant reduction in macrophages (by 38%) but not in lymphocyte and granulocyte populations compared with those of control Ab-treated mice (Fig. 6A).

In separate experiments, we evaluated the total lung-infiltrating leukocytes dispersed by collagenase (Fig. 6B). The total number of infiltrating leukocytes in anti-CCR1 Ab-treated mice was significantly decreased (by 42%) compared with that of control Ab-treated mice. Differential counts of infiltrating leukocytes were performed with immunofluorescence analyses. The number of CD4,CD11c-positive cells was significantly decreased, whereas the number of CD11b,CD8,Gr-1-positive cells tended to be decreased but did not reach significant differences.

Discussion

Pulmonary fibrosis has been treated with corticosteroids or immunosuppressants with little outcome of improvement. Analysis of molecular mechanisms can provide more effective and novel therapeutic targets. Our results suggest that CCR1-positive cells play an essential role in the pathogenesis of pulmonary fibrosis, and CCR1 could be a potent molecular target. In bleomycin-induced lung injury, a model of pulmonary fibrosis, epithelial cell injury and alveolar inflammation occur first, followed by interstitial inflammation, which results in pulmonary fibrosis. There may be at least three conceivable approaches to block pulmonary fibrosis. First, to avoid the epithelial injury, Fas-Fas ligand pathway could be one of the useful targets (25). Second, to control the fibrotic phase, TGF-β pathway, including integrin αVβ6, could be a major target (6, 7). Third, to control the unremitting inflammation characterized by leukocyte infiltration may be one of the hopeful therapeutic approaches. To control inflammation, cytokines or chemokines could be potent molecular targets. Although neutralization of TNF-α, MIP-2, or MIP-1α improved fibrotic changes (5, 8, 10), the role of their receptors in regulating inflammation and fibrosis so far remains unclear. The specific chemokine receptor expression and regulation on each type of leukocyte may potentially control the selective leukocyte recruitment and activation at the inflammatory site (26, 27). Although MIP-1α and RANTES have been
implicated in the pathogenesis of pulmonary fibrosis, our data suggest that their common receptor CCR1 will be a novel therapeutic target in this disease.

After bleomycin challenge the expression of CCR1 mRNA as well as its ligands MIP-1α and RANTES mRNA increased and peaked at day 7 (Fig. 2). Immunohistochemical study (Fig. 3) and immunofluorescence analysis (Fig. 1) showed that CCR1-positive cells may be mainly macrophages and dendritic cells accumulated in the subepithelial and interstitial areas (Fig. 3, a–c). Although involvement of macrophages and lymphocytes was reported, the contribution of dendritic cells has not been explored. We recognized NLDC-145- or MHC class II-positive cells in the subepithelial and interstitial areas after bleomycin challenge (Fig. 3, h and i). Immature dendritic cells are attracted toward the inflammatory site by MIP-1α, RANTES, and MIP-3α through their receptors CCR1 and CCR6. Dendritic cells are the sources of activated T cell chemotaxtractants, such as EBI-1 ligand chemokine, thymus and activation-regulated chemokine, and macrophage-derived chemokine (19). After anti-CCR1 Ab treatment, the number of CD4+ as well as CD11c-positive cells was reduced (Fig. 6B). These results suggest that dendritic cells expressing CCR1 might play a role in maintaining chronic inflammation including pulmonary fibrosis.

Anti-CCR1 Ab treatment significantly improved the survival in association with the reduction of collagen deposition (Figs. 4 and 5). Anti-CCR1 Ab treatment reduced the total cell count of BAL fluid as well as the infiltrating leukocyte count in tissue (Fig. 6). Smith et al. (8) reported that in the bleomycin model neutralization of MIP-1α reduced the macrophage accumulation without modulating other leukocytes such as lymphocytes or granulocytes. Our data showed that anti-CCR1 Ab treatment reduced not only the number of macrophages in BAL fluid but also other leukocyte subpopulations (Fig. 6). The participation of other ligands of CCR1 such as RANTES and leukotactin-1 could explain the difference in the effects of anti-MIP-1α Ab and anti-CCR1 Ab in this model. In CCR1-deficient mice, the size of Schistosoma mansoni-induced granuloma was reduced, but there were no differences in the cellular composition of the granuloma (28). These data suggest a possible effect of CCR1 on leukocyte migration for many leukocyte subtypes in vivo.

Compared with CCR1Ab treatment, neutralization of CCR2 failed to modulate fibrotic responses (Figs. 4B and 5B), although its ligand MCP-1/JE may participate in this pathogenesis (Fig. 2A). Immunofluorescence analysis of CCR2 showed a similar distribution pattern to CCR1, i.e., the majority of CD11b-positive cells were strongly stained and CD3-positive cells were weakly stained with anti-CCR2 Ab (data not shown). Failure of anti-CCR2 Ab treatment in this model in spite of the similar distribution pattern might be considered as follows. First, the timing of the Ab treatment might not be appropriate. However, in our preliminary experiments, administration of anti-CCR2 Ab at every 2 days from days 0 to 12 failed to improve the survival rate and collagen deposition (data not shown). This result may be able to exclude the possibility of the inappropriate treatment. Second, other chemokine receptors on the CCR2-expressing cells might compensate; therefore, anti-CCR2 Ab could not block the migration of the CCR2- expressing cells in vivo. However, CCR2-deficient mice failed to recruit macrophages in an experimental peritoneal inflammation (29), suggesting that CCR2 has a nonredundant role. Third, the MIP-1α–CCR1 pathway plays more of an essential role than the MCP-1–CCR2 pathway does in this model. CCR1 and CCR2 could be expressed on the same cells. Although both receptors participate in leukocyte chemotaxis and activation, they might act in different way. In bleomycin-induced lung injury, anti-MIP-1-α Ab reduced the total number of CD11b-positive cells and showed significant reduction of fibrosis (1). Although anti-MCP-1 Ab also reduced total lung inflammatory cell count, there has been no reports demonstrating an antifibrotic effect (1). Chemokine receptor-deficient mice would provide additional information. In CCR1-deficient mice, the size of S. mansoni-induced granuloma was reduced (28). In contrast, in CCR2-deficient mice, the granuloma size could not be reduced, although the size and macrophage number were reduced only in the early phase (30).

Despite chemokine redundancy, the neutralization of a single chemokine receptor could affect a remarkable decrease in inflammatory responses and fibrotic responses. There are two considerable explanations. First, the neutralization of CCR1 results in an overall decrease of CCR1-expressing leukocyte subpopulations, including monocytes, lymphocytes, neutrophils, eosinophils, and immature dendritic cells. Second, neutralization of CCR1 would initially lead to a decrease of the infiltration and activation in the CCR1-expressing key cells, such as alveolar macrophages (Fig. 3c), which are the potentially important source of cytokines and chemokines, including TNF-α, MIP-1α, RANTES, and MCP-1/JE (1, 4, 8, 9, 11, 12). Anti-CCR1 Ab reduced the alveolar macrophages in number by 38% (Fig. 6A), which may cause the reduction of these cytokines and chemokines resulting in a remarkable decrease in the inflammatory responses, through blocking the autocrine process. Gerard et al. (31) reported that in pancreatitis-associated lung injury, CCR1-deficient mice did not show any increase in TNF-α levels in BAL fluid. Neutralization of the chemokine receptor could modulate not only direct leukocyte migration but also secondary response.

In this report, we detected time-dependent expression of CCR1 mRNA and CCR1-positive cells accumulated in the inflammatory site, demonstrating that chemokine receptor CCR1 contributes to the inflammatory responses in bleomycin-induced lung injury. Furthermore, anti-CCR1 Ab treatment in bleomycin-challenged mice significantly improved the survival rate and decreased collagen deposition, corresponding with the reduction of inflammatory cell accumulation. Anti-CCR1 Ab treatment failed to achieve the complete inhibition of both inflammatory cell infiltrations into lung and subsequent fibrosis, suggesting that other chemokine receptors are also involved in the pathogenesis of the disease. Nevertheless, our data suggest that chemokine receptors could be a potent therapeutic target against pulmonary fibrosis. Additional experiments are also expected to establish the therapeutic possibility of targeting CCR1 in other inflammatory diseases involving MIP-1α and RANTES such as rheumatoid arthritis and multiple sclerosis.

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