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Protection from Pulmonary Fibrosis in the Absence of CCR2 Signaling

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Pulmonary fibrosis can be modeled in animals by intratracheal instillation of FITC, which results in acute lung injury, inflammation, and extracellular matrix deposition. We have previously shown that despite chronic inflammation, this model of pulmonary fibrosis is lymphocyte independent. The CC chemokine monocyte-chemoattractant protein-1 is induced following FITC deposition. Therefore, we have investigated the contribution of the main monocyte-chemoattractant protein-1 chemokine receptor, CCR2, to the fibrotic disease process. We demonstrate that CCR2−/− mice are protected from fibrosis in both the FITC and bleomycin pulmonary fibrosis models. The protection is specific for the absence of CCR2, as CCR5−/− mice are not protected. The protection is not explained by differences in acute lung injury, or the magnitude or composition of inflammatory cells. FITC-treated CCR2−/− mice display differential patterns of cellular activation as evidenced by the altered production of cytokines and growth factors following FITC inoculation compared with wild-type controls. CCR2−/− mice have increased levels of GM-CSF and reduced levels of TNF-α compared with FITC-treated CCR2+/+ mice. Thus, CCR2 signaling promotes a profibrotic cytokine cascade following FITC administration. The Journal of Immunology, 2001, 167: 4368–4377.

Diopathic pulmonary fibrosis (IPF) is the consequence of a number of diverse insults that result in damage to the alveolar surface of the lung. The disease process is characterized by alveolar epithelial cell injury and hyperplasia, inflammatory cell accumulation, fibroblast hyperplasia, and deposition of extracellular matrix with scar tissue formation. The end result of this process is the loss of lung elasticity and loss of alveolar surface area leading to impairment of gas exchange and severe compromises in pulmonary function. The orchestration of injury, inflammation, and the molecules that mediate the pathogenesis of this disease is largely unknown. Chronic inflammation is a nearly universal feature of human IPF and is postulated to play a central role in the fibrotic response. An interaction between resident or recruited inflammatory cells and structural cells likely results in chronic inflammatory infiltrates, myofibroblast hyperplasia, and disordered collagen deposition. Chemokines are likely candidates to serve as mediators of chronic inflammation in IPF. Furthermore, they may play roles in cellular activation. One chemokine that is involved in these processes is monocyte chemoattractant protein-1 (MCP-1), also known as JE in the mouse. MCP-1 was first identified as a platelet-derived growth factor-inducible product (JE) from murine 3T3 fibroblasts. MCP-1 synthesis has been detected in endothelial cells, epithelial cells, fibroblasts, monocyte/macrophages, vascular smooth muscle cells, keratinocytes, and pulmonary mast cells. MCP-1 is expressed in numerous inflammatory conditions including persistent acute respiratory distress syndrome (ARDS), IPF, atherosclerosis, and crescentic nephritis.

We have recently characterized the use of intratracheal FITC challenge as a model of lung injury that results in lymphocyte-independent pulmonary fibrosis. FITC challenge results in acute lung injury, chronic inflammation, and the eventual deposition of extracellular matrix and scar tissue formation. This protocol results in areas of patchy inflammation and subpleural scarring characteristic of animal models of pulmonary fibrosis. In response to this insult, numerous cytokines, chemokines, and growth factors are produced that mediate the fibrotic/repair process. We demonstrate that the pro-fibrotic signaling cascade is diminished in CCR2−/− mice in two different models, suggesting that CCR2 signaling is part of a generalized pathway leading to pulmonary fibrosis in response to diverse insults.
Materials and Methods

Mice

CCR2<sup>−/−</sup> (B6129F2/J; The Jackson Laboratory, Bar Harbor, ME), CCR2<sup>−/−</sup> (B6129F2-Cmkbr<sup>2m1Kuz</sup>), and CCR5<sup>−/−</sup> (B6129F2-Cmkr<sup>5m1Kuz</sup>) were bred at the University of Michigan, were housed under specific pathogen-free conditions in enclosed top cages. Clean food and water was given ad libitum. The mice were handled and maintained using microisolator techniques with daily veterinarian monitoring. The University of Michigan Committee on the Use and Care of Animals approved these experiments.

FITC and bleomycin injections

CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> mice were anesthetized with sodium pentobarbita
tal. The trachea was exposed and entered with a needle under direct visu-
alization. For experiments using FITC injection, 21 mg of FITC (F-7250; Sigma, St. Louis, MO) was dissolved in 10 ml of sterile PBS, vortexed extensively, and sonicated for 30 s. This slurry was transferred to multiuse vials, and vortexed extensively before each 50-μl aliquot was removed for intratracheal injection using a 26-gauge needle. For bleomycin experi-
ments, a single 30-μl injection containing 0.025 U of bleomycin (Sigma) diluted in normal saline was injected using a Triad stepper (Brookfield, CT) and a 30-gauge needle.

Hydroxyproline assays

Mice were euthanized by CO<sub>2</sub> asphyxiation and perfused via the heart with 5 ml of normal saline. Individual lung lobes were removed, taking care to avoid the large conducting airways. The isolated lobes were homogenized in 1 ml of PBS, and hydrolyzed by the addition of 1 ml of 12 N hydro-
chloric acid (HCl). Samples were then baked at 110° C for 12 h. Aliquots (5 μl) were then assayed by adding chloramine T solution for 20 min followed by development with Erlich’s reagent at 65° C for 15 min as previously described (29). Absorbance was measured at 550 nm, and the amount of hydroxyproline was determined against a standard curve gen-
erated using known concentrations of hydroxyproline standard (Sigma).

Histology

Animals were euthanized and perfused via the right ventricle with 3 ml of normal saline. Lungs were inflated with 1 ml of 10% neutral buffered formalin, removed, and fixed overnight in formalin before being dehy-
drated in 70% ethanol. Lungs were processed using standard procedures and embedded in paraffin. Sections (3–5 μm) were cut, mounted on slides, and stained with H&E or Masson’s trichrome blue for collagen deposition.

Evans blue permeability assays

Lung permeability was determined by assessing tissue accrual of Evans blue as previously described (30). This assay measures the leak of Evans blue dye, injected into the tail vein 30 min before sacrifice, into pulmonary tissue. Thus, the ratio of dye found in the lung vs the plasma is a measure of vascular permeability and acute lung injury. Animals were administered 20 mg/kg Evans blue (Sigma) by tail vein injection 30 min before lung harvest. Lungs were perfused with 1 ml of PBS + 5 mM EDTA, followed by removal and snap-freezing in liquid nitrogen. Samples were homoge-
nized in 2 ml of PBS. Evans blue was extracted from lung homogenates by incubating the sample in 2 volumes of formamide at 60° C for 18 h. The supernatant was then separated by centrifugation at 5000 × g for 30 min. Evans blue concentration in lung homogenate supernatants was quantitated by a dual wavelength spectrophotometric method at absorbances of 620 and 740 nm, which allows for correction of contaminating heme pigments, as determined by the following formula: E<sub>620</sub> (Evans blue) = E<sub>620</sub> – (1.426 × E<sub>740</sub> + 0.030). Data are presented as tissue value normalized to plasma value.

Bronchoalveolar lavage (BAL) and determination of protein concentration

Mice were euthanized via CO<sub>2</sub> asphyxiation, the trachea cannulated with polyethylene tubing (PE50, Intramedic; Clay Adams, Parsippany, NJ) at-\tached to a 25-gauge needle on a tuberculin syringe, and the lungs were lavaged twice with 0.75 ml of PBS/5 mM EDTA (Sigma) for a total lavage volume of 1.5 ml. In >95% of the mice, the recovery volume was 1.3–1.4 ml. The BAL fluid was spun at 1500 rpm, and the supernatant was re-
moved. Total protein content in BAL fluid was measured using a modifi-
cation of the Bradford protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

Collagenase digestions of whole lung

Collagenase digestions can be used to analyze both resident and recruited populations of lung cells found both in the alveolar space and interstitium. This procedure has been optimized to purify lung leukocytes (31). Lungs were excised, minced, and enzymatically digested for 30 min using 15 ml/lung of digeston buffer (RPMI 1640, 5% FCS, antibiotics, 1 mg/ml collagenase (Boehringer-Mannheim, Chicago, IL) and 30 μg/ml DNase (Sigma)). The cell suspension and undigested fragments were further dis-
persed by repeated passage through the bore of a 10-ml syringe by a needle. The total cell suspension was pelleted, and any contaminating erythrocytes were eliminated by lysis in ice-cold NH<sub>4</sub>Cl buffer (0.829% NH<sub>4</sub>Cl, 0.1% KHCO<sub>3</sub>, and 0.0372% Na<sub>2</sub>EDTA, pH 7.4). The pellet was resuspended in 5 ml of complete medium (RPMI 1640, 5% FCS, 1% pen-
nicillin/streptomycin) and dispersed by 20 passages through a 5-ml syringe. The dispersed cells were filtered through a Nitex filter (Tekton, Kansas City, MO) to remove clumps. The total volume was brought up to 10 ml with complete medium. An equal volume of 40% Percoll (Sigma) was added, and the cells were centrifuged at 3000 rpm for 30 min (room tempe-
ration) without a brake. The cell pellets were resuspended in complete medium, and leukocytes were counted on a hemacytometer in the presence of trypsin blue. Cells were >90% viable by trypan blue exclusion. Cyto-
spins of recovered cells were prepared for differential staining as described below. In addition, recovered leukocytes were analyzed by flow cytometry.

Differential staining

Cytospins of collagenase digestions were made by centrifuging 50,000 cells onto microscope slides using a Shandon Cytospin 3 (Astmoore, En-
gland). The slides were allowed to air dry and were stained using a mod-
dification of the May-Grunwald–Giemsa (Wright-Giemsa) stain. For WG staining, the slides were fixed and pre-stained for 2 min with a one-step methanol-based WG stain (Harleco; EM Diagnostics, Gibbstown, NJ), followed by steps 2 and 3 of the Diff-
Quick whole blood stain (Diff-Quick; Baxter Scientific, Miami, FL). This modification of the Diff-Quick stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 300 cells were counted from randomly chosen high power microscope fields for each sam-
ple. The differential percentage was multiplied by the total leukocyte num-
ber to derive the absolute number of monocyte/macrophages, neutrophils, and eosinophils per sample.

FACS analysis

Lung cells (1 × 10<sup>6</sup>) from the collagenase digestions of individual animals were incubated for 15 min on ice in Fc block (BD Pharamingen, San Diego, CA) before washing and centrifugation. Cells were stained in a 100-μl total volume with 1-μg combinations of the following Abs (obtained from BD Pharamingen unless otherwise noted): CD45 (YW62.5; Caltag Labora-
tories, Burlingame, CA), CD4 (RM4-4), CD8 (53-6.7), CD19 (1D3), or DX5. Stained samples were stored in the dark at 4°C until analyzed on a flow cytometer (FACSscan; BD Biosciences, Mountain View, CA). All samples were stained with CD45 to identify a leukocyte-specific gate. The absolute number of a type of leukocyte in the lungs was determined as the percent-
age of that cell type times the total number of cells (leukocytes) in the lung collagenase digestion. Mean data represent the averages of three indepen-
dent experiments with a total of eight animals per group.

Lung RNA preparation

Whole lungs were perfused with saline, removed, snap-frozen in liquid nitrogen, and stored at −70°C until use. To prepare RNA, frozen lungs were homogenized in 3 ml of TRIzol reagent (Life Technologies, Gaith-
ersburg, MD) and extracted according to manufacturer’s instructions.

RNase protection analysis of lung mRNA

RNase protection assays for lung GM-CSF and TNF-α mRNA were per-
formed using pooled total RNA from mice treated with FITC. GM-CSF was assayed with the Riboquant CK-1 probe set, and TNF-α was assayed with the CK-3b probe set (both obtained from BD Pharamingen) according to manufacturer’s recommendations for hybridization, digestion, and elec
trophoresis. GAPDH quantification was performed on autoradiographs de-
veloped from 1- to 3-h exposures, whereas GM-CSF and TNF-α quantifi-
cations were from 24-h exposures.

Densitometric analyses

A digital picture of each autoradiograph was taken, and band intensities were analyzed using NIH Image public domain software (developed at the Research Services Branch of the National Institute of Mental Health; available for download at http://rsb.info.nih.gov/nih-image). Specific cytokine
band intensities were normalized to GAPDH controls to account for differences in total RNA loading in each sample.

Lung homogenates

Lung homogenates were prepared from experimental animals at indicated time points. Animals were euthanized and perfused with 3 ml of PBS via the heart. Isolated lung lobes were removed and snap-frozen in liquid nitrogen until analysis. Lung lobes were homogenized using a Tissue Tearor (Biospec Products, Bartlesville, OK) at setting 5 in 1 ml of PBS containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Samples were then sonicated for 10 s before cellular debris was removed by centrifugation for 10 min at 3000 rpm. Homogenates were then filtered through a 0.45 μm filter before protein analysis via ELISA. In addition, protein concentrations within lung homogenates were determined using the Bio-Rad protein assay (Hercules, CA) according to manufacturer’s instructions.

ELISA

The measurements for MCP-1, GM-CSF, and TNF-α were performed on lung homogenates using Opti-EIA kits obtained from BD PharMingen according to manufacturer’s instructions. Sensitivity ranged from 10 to 50 pg/ml at the lower limit.

Statistics

Statistical significance was analyzed using the InStat 2.01 program (GraphPad Software) on a Power Macintosh G3. Student’s t tests were run to determine p values when comparing two groups. When comparing three or more groups, ANOVA analysis was performed with a post hoc Bonferroni test to determine which groups showed significant differences. Values of p < 0.05 were considered significant.

Results

MCP-1 is induced following FITC injury

We have previously shown that mixtures of both acute and chronic inflammatory cells persist in fibrotic areas following FITC administration (26). Therefore, we reasoned that mononuclear chemotactic factors might be elevated. To determine whether a mononuclear chemotactic factor was being induced following FITC injury, we examined lung homogenates at various time points post-FITC inoculation for MCP-1 protein accumulation. Fig. 1A documents that MCP-1 is elevated in the CCR2+/+ wild-type animals at days 1 and 3. In addition, we analyzed CCR2−/− animals for MCP-1 induction following FITC administration (Fig. 1B). Higher levels of MCP-1 ligand are noted in the absence of the CCR2 receptor compared with CCR2+/+ mice. We analyzed lung homogenates at days 7, 10, 14, and 21 as well, but did not detect elevated levels of MCP-1 compared with baseline by ELISA at these later time points in wild-type or CCR2−/− animals. These data demonstrate that MCP-1 is induced following FITC-mediated acute lung injury in both CCR2+/+ and CCR2−/− mice. The expression of the other murine CCR2 ligands, MCP-3 and MCP-5, was analyzed by RT-PCR. MCP-3 is not induced following FITC administration, but MCP-5 mRNA is induced ~2-fold at day 7 post-FITC compared with baseline (data not shown).

CCR2−/− mice are protected from pulmonary fibrosis

Given the elevated expression of MCP-1 following FITC administration, we hypothesized that MCP-1 signaling via its receptor, CCR2, is involved in the fibrotic response to FITC. Therefore, we examined the consequences of FITC administration in CCR2−/− mice. CCR2−/− mice are protected from pulmonary fibrosis, compared with CCR2−/− mice. We analyzed lung homogenates at days 7, 10, 14, and 21 as well, but did not detect elevated levels of MCP-1 compared with baseline by ELISA at these later time points in wild-type or CCR2−/− animals. These data demonstrate that MCP-1 is induced following FITC-mediated acute lung injury in both CCR2+/+ and CCR2−/− mice. The expression of the other murine CCR2 ligands, MCP-3 and MCP-5, was analyzed by RT-PCR. MCP-3 is not induced following FITC administration, but MCP-5 mRNA is induced ~2-fold at day 7 post-FITC compared with baseline (data not shown).

Figure 1. A, CCR2−/− mice are protected from fibrosis induced by FITC or bleomycin. A, CCR2+/+ or CCR2−/− mice were injected with either saline or FITC, and lungs were harvested at day 21 and measured for hydroxyproline content as a measure of collagen deposition. CCR2−/− mice are significantly protected from FITC-induced pulmonary fibrosis compared with CCR2+/+ FITC-injected mice (p = 0.03). Data represent the analysis of eight mice per group, representative of three separate experiments. B, Same experiment performed using bleomycin as the stimulus to generate pulmonary fibrosis. CCR2−/− mice were also protected from bleomycin-induced pulmonary fibrosis, compared with CCR2+/+ mice (p = 0.0003). Data represent the analysis of six mice per group, representative of two separate experiments.
mice compared with wild-type, CCR2<sup>+/+</sup> mice. To quantitatively determine the extent of fibrosis induced by FITC, we measured hydroxyproline as a surrogate for lung collagen deposition. On day 21 post-FITC, CCR2<sup>+/+</sup> mice display higher levels of hydroxyproline compared with CCR2<sup>−/−</sup> mice (Fig. 2A) on day 21 (<i>p</i> = 0.03). There were no significant differences in the baseline levels of collagen found in CCR2<sup>+/+</sup> or CCR2<sup>−/−</sup> mice injected with saline. Masson’s Trichrome staining of day 21 post-FITC lungs in CCR2<sup>+/+</sup> and CCR2<sup>−/−</sup> mice was also performed (see Fig. 3, bottom panel). The blue staining represents areas of mature collagen deposition. This analysis confirms that CCR2<sup>−/−</sup> mice show attenuated levels of collagen deposition. To determine whether the protection was limited to FITC, the fibrotic response to bleomycin in CCR2<sup>+/+</sup> and CCR2<sup>−/−</sup> mice was also studied.

As seen in Fig. 2B, CCR2<sup>−/−</sup> mice are similarly protected from bleomycin-induced pulmonary fibrosis. The bleomycin-treated CCR2<sup>−/−</sup> mice developed significantly less pulmonary fibrosis (<i>p</i> = 0.0003) compared with bleomycin-treated CCR2<sup>+/+</sup> mice. Thus, CCR2<sup>−/−</sup> mice are protected from pulmonary fibrosis in two independent models.

**CCR5<sup>−/−</sup> mice are not protected from pulmonary fibrosis**

To determine whether the protection seen in CCR2<sup>−/−</sup> mice was specific for a particular CCR, we analyzed FITC-induced pulmonary fibrosis in CCR5<sup>−/−</sup> mice. CCR5 is the receptor for the CC chemokines macrophage-inflammatory protein (MIP)-1α and RANTES, both of which are mononuclear cell chemotaxins. Fig. 4A demonstrates that CCR5<sup>−/−</sup> mice show equivalent hydroxyproline accumulation following FITC injection as wild-type mice, whereas CCR2<sup>−/−</sup> mice show more extensive fibrosis at day 21 compared with CCR2<sup>−/−</sup> mice.

**FIGURE 3.** Histology from CCR2<sup>+/+</sup> and CCR2<sup>−/−</sup> mice at days 0, 1, 3, 7, and 21 following FITC. Mice were sacrificed at the time points indicated following FITC administration, and histologic sections were prepared and stained with H&E on days 0–21 (×20 magnification). On day 1, similar levels of exudate were seen in the lungs of both strains of mice following FITC. On day 3, similar levels of alveolar hemorrhage were seen, and at day 7, similar levels of inflammatory cell accumulation and exudate were noted, suggesting levels of acute lung injury that were similar between the two strains of mice in response to FITC. On day 21, lungs were harvested for histological analysis using H&E staining (×20 magnification) or Masson’s Trichrome stain (×40 magnification) for mature collagen deposition. Histology shown is representative of six mice examined at each time point through day 7. Histologic pattern shown is representative of all mice examined within a particular group. Day 21 histology is representative of 15 animals examined. As confirmed by hydroxyproline analysis, all CCR2<sup>+/+</sup> mice show more extensive fibrosis at day 21 compared with CCR2<sup>−/−</sup> mice.
analysis of bleomycin-induced pulmonary fibrosis revealed that only CCR2−/− mice are protected (p = 0.0003), whereas the wild-type and CCR5−/− mice are susceptible to bleomycin-induced pulmonary fibrosis (Fig. 4B). Thus, the protection from fibrotic insults is specific to the absence of the CCR2 receptor-signaling pathway.

FITC-induced acute lung injury is similar in wild-type and CCR2−/− mice

One explanation for the protection seen in CCR2−/− mice following FITC injection would be that the degree of initial lung injury in CCR2−/− mice following FITC deposition is less than in wild-type mice. We examined histology sections from lungs of mice at days 0, 1, 3, 7, and 21 following FITC injection. The histologic pattern of injury seen in CCR2+/+ and CCR2−/− mice is indistinguishable throughout day 7 (Fig. 3). Both CCR2+/+ and CCR2−/− mice show evidence of alveolar fluid accumulation at day 1 and inflammation and hemorrhage at days 3 and 7. Thus, it appears that the acute lung injury phase of the fibrotic process is similar between CCR2+/+ and CCR2−/− mice. However, when histological sections are examined at day 21 post-FITC, CCR2+/+ mice demonstrate areas of mononuclear cell inflammation and consolidation reported earlier for wild-type mice (26). These areas are much less pronounced in the CCR2−/− mice. This histological evidence suggests that despite similar early injury, the fibrotic process is minimized in the CCR2−/− mice.

To determine quantitatively the level of acute lung injury, CCR2+/+ and CCR2−/− mice were injected with FITC, and BAL fluid was collected at days 1, 3, and 7 following FITC inoculation. Lung permeability increases when alveolar epithelial and endothelial cells are damaged, allowing fluid to accumulate in the alveolar space. Therefore, total protein concentrations in the BAL fluid were determined as a measure of plasma leak into the alveolar space. BAL fluid protein increased dramatically 1 day post-FITC, confirming equivalent early leak in both CCR2+/+ and CCR2−/− mice (Fig. 5A). At day 3, the protein concentration in the CCR2−/− mice is less than that seen in the CCR2+/+ mice (p = 0.03), but by day 7, the protein concentrations in the BAL were again equivalent.

To further determine the vascular permeability induced by FITC deposition, we performed an Evans blue extravasation assay at day 7 following FITC administration. A, CCR2+/+ and CCR2−/− mice were injected with FITC, and BAL samples were collected and analyzed for total protein concentrations. FITC inoculation induced acute lung injury as measured by protein accumulation in the BAL. The levels of protein leak were similar at days 1 and 3; however, the CCR2−/− mice showed a lower protein level at day 3. Data represent the analysis of three animals per time point, representative of two separate experiments. B, Vascular permeability in the lung following FITC administration was evaluated by injecting CCR2+/+ or CCR2−/− mice with FITC on day 0. On days 0, 1, 3, and 7 post-FITC, BAL fluid protein increased dramatically 1 day post-FITC, confirming equivalent early leak in both CCR2+/+ and CCR2−/− mice (Fig. 5A). At day 3, the protein concentration in the CCR2−/− mice is less than that seen in the CCR2+/+ mice (p = 0.03), but by day 7, the protein concentrations in the BAL were again equivalent.
days 0, 1, 3, and 7 following FITC injection. Fig. 5B demonstrates that the vascular permeability seen in CCR2+/+ and CCR2−/− mice was similar at all time points tested following FITC inoculation. Both groups of mice show evidence of similar acute lung injury early following FITC administration that then returns to baseline in both groups of mice. Thus the protection seen in CCR2−/− mice cannot be explained by differences in early lung injury as measured by capillary leak or edema.

The inflammatory response to FITC is similar in CCR2+/+ and CCR2−/− mice

Having determined that the protection seen in CCR2−/− mice could not be attributed to differences in acute lung injury induced by FITC, we examined whether the inflammatory response was different in the two groups of FITC-treated mice. The magnitude and composition of the inflammatory response was determined by performing collagenase digestion and leukocyte purification on whole lungs from treated animals at days 0, 7, 14, and 21. At baseline, both CCR2+/+ and CCR2−/− mice show equivalent numbers of resident leukocytes in their lungs (14.08 and 14.02 × 10⁶, respectively). At day 7, there is a significant increase in the number of leukocytes compared with baseline in both groups of mice (24.22 × 10⁶, p = 0.0001 in the CCR2+/+ and 25.13 × 10⁶, p = 0.0001 in the CCR2−/− animals). By day 14, the number of leukocytes in the CCR2+/+ mice returned to baseline (14.84 × 10⁶); however, leukocyte cell number was still modestly elevated compared with baseline in the CCR2−/− mice (19.7 × 10⁶, p = 0.009). By day 21, the cell numbers in both CCR2+/+ and CCR2−/− mice are not statistically different from baseline levels (16.01 × 10⁶ in the CCR2+/+ and 17.7 × 10⁶ in the CCR2−/− mice). In all cases, SE values were <10% and represent data pooled from eight mice in three separate experiments.

To determine whether the composition of the inflammatory response to FITC differed between the CCR2+/+ and CCR2−/− mice, flow cytometry and differential analyses were performed to identify leukocyte subpopulations. Fig. 6 demonstrates that there were no statistical differences between any of the leukocyte subpopulations analyzed between CCR2+/+ and CCR2−/− mice at the peak of inflammation (day 7). Similarly, no differences were noted in the composition of the inflammatory cells at days 14 or 21 (data not shown). Fig. 7 shows representative staining profiles of the

**FIGURE 6.** Absolute levels of leukocyte subpopulations in the lung at day 7 post-FITC. CCR2+/+ and CCR2−/− mice were injected with FITC at day 0, and lungs were harvested at day 7 for differential counting and flow cytometry analysis to characterize the leukocyte subpopulations at the peak of inflammation following FITC. Following collagenase digestion, cytopsins were prepared and stained for differential analysis. Three hundred cells were counted from randomly chosen high-power fields and identified as being neutrophils, eosinophils, or monocyte-macrophages. The percentage of each was multiplied by the total number of leukocytes in the collagenase digestion to determine the absolute number. The lymphocyte subpopulations were analyzed by flow cytometry. Collagenase digestions were stained with CD45 to identify the leukocyte-specific cells, and then were gated on lymphocyte-sized cells to be analyzed for subset markers (see Fig. 7). There were no statistical differences in any subpopulation analyzed between the CCR2+/+ and CCR2−/− mice. Data represent mean ± SEM values of eight animals per group.

**FIGURE 7.** Representative FACs analysis of CCR2+/+ and CCR2−/− mice at day 7 post-FITC. Collagenase-digested lung cells were first incubated with Fc block on ice for 15 min before staining with other markers. All samples were stained for CD45 (TriColor) and then costained with specific subset markers labeled with PE. **Top left panel,** Representative dot plot of a collagenase digest in a CCR2+/+ mouse treated with FITC. **Top right panel,** Demonstrates the lymphocyte-specific gate that is drawn on the basis of size (side scatter vs forward scatter). Both patterns are identical in CCR2−/− animals (data not shown). **Lower panels,** Representative staining from a CCR2+/+ and a CCR2−/− mouse treated with FITC. Histograms represent specific PE staining of indicated lymphocyte subset markers within the lymphocyte gate. Dark line indicates specific marker staining, and light line represents Ab control staining. M2 marker region distinguishes cells that were identified as being positive, and represents percent positive within the leukocyte gate. Absolute numbers of a given subpopulation were calculated by multiplying the percentage of positive cells times the total number of leukocytes in the collagenase digestion. Data are representative of eight animals per group.
lymphocyte-gated population analyzed in CCR2+/+ and CCR2−/− mice at day 7 post-FITC. The staining profiles (percentage of positive cells, and the relative fluorescent intensity of positive cells) are similar in both groups. The same was true of the analyses at days 14 and 21 (data not shown). Thus, the protection in CCR2−/− mice cannot be explained by differential recruitment of inflammatory cells.

The cytokine cascade following FITC inoculation is altered in CCR2−/− mice

The differences in the fibrotic response between CCR2+/+ and CCR2−/− mice could not be explained by differences in the magnitude or the composition of the cellular recruitment. To determine whether differences in cellular activation were responsible for the protection of the CCR2−/− mice, we analyzed the expression of two molecules known to be involved in early inflammatory responses and fibrotic processes: TNF-α and GM-CSF. We chose to evaluate the levels of TNF-α and GM-CSF at day 14 post-FITC inoculation. At this time point, fibroproliferative changes are evident as measured by collagen deposition. We analyzed total lung RNA from CCR2+/+ and CCR2−/− mice at day 14 post-FITC to quantitate mRNA for TNF-α and GM-CSF using ribonuclease protection assays. Fig. 8 shows the results of these assays. Densitometry analysis was performed, and cytokine-specific mRNA levels were normalized to GAPDH control mRNA levels. These analyses demonstrate that, collectively, GM-CSF mRNA levels are ~3 times higher in CCR2−/− mice compared with CCR2+/+ mice treated with FITC. Conversely, levels of TNF-α mRNA are ~11 times higher in CCR2−/− mice compared with CCR2+/+ mice at day 14 post-FITC.

To confirm that these cytokine imbalances were evident at the protein level, and also were maintained at later time points in the fibrotic process, we analyzed lung homogenates from CCR2+/+ and CCR2−/− mice for expression of TNF-α and GM-CSF at day 21 post-FITC. Fig. 9 shows the results of these experiments, and verifies that TNF-α protein levels are still elevated in CCR2−/− mice at day 21 (Fig. 9A), whereas GM-CSF levels are elevated in CCR2−/− mice at day 21 (Fig. 9B). Thus, the imbalance in pro- and anti-fibrotic mediators is maintained at later time points.

**FIGURE 8.** Fibrotic signaling cascade is altered in CCR2+/+ and CCR2−/− mice on day 14 post-FITC. GM-CSF and TNF-α mRNA levels were analyzed using RNase protection assays. Each lane represents the pooled RNA (15 μg) from three individual mice (5 μg each) treated with FITC and harvested at day 14. Total RNA was hybridized to radioactively labeled CK-1 oligo template for GM-CSF, and radiolabeled CK3b template for TNF-α according to the manufacturer’s recommendations using the BD PharMingen Riboquant RNase protection assay kits. Shown are the protected species corresponding to GM-CSF, TNF-α, and their respective control gene GAPDH hybridizations. When normalized for GAPDH, the expression of GM-CSF is elevated 3-fold in the CCR2−/− mice compared with CCR2+/+ mice. When normalized to GAPDH, the expression of TNF-α is elevated almost 11-fold in CCR2−/− mice compared with CCR2+/+ mice.

**FIGURE 9.** Cytokine imbalances are maintained at day 21 post-FITC. CCR2+/+ and CCR2−/− mice were injected with FITC at day 0, and lung homogenates were harvested at day 21 to determine protein concentrations for GM-CSF and TNF-α. Bars represent the analysis of six animals per group, and the cytokine levels were normalized to total protein levels in the homogenate. A, CCR2−/− mice have significantly higher levels of TNF-α present in lung homogenates at day 21 (p = 0.0062) compared with CCR2+/+ mice. Conversely, the levels of GM-CSF are significantly elevated in CCR2−/− mice at day 21 (p = 0.02) compared with CCR2+/+ mice.

**Discussion**

Our studies demonstrate that signaling via the CCR2 receptor leads to the generation of pro-fibrotic signals following FITC or bleomycin inoculation. In particular, our studies yield several important points: 1) in the absence of CCR2, FITC-induced pulmonary fibrosis is diminished both histologically and quantitatively; 2) the protection is related to the absence of this specific chemokine receptor; deletion of the CCR5 receptor had no effect on the severity of pulmonary fibrosis; 3) the protection conferred by the absence of CCR2 is a generalized phenomenon; CCR2−/− mice are protected from pulmonary fibrosis induced by either FITC or bleomycin; 4) the protection from pulmonary fibrosis seen in CCR2−/− mice is not due to differences in early lung injury caused by the FITC inoculation; 5) despite the absence of the CCR2 receptor, CCR2−/− mice develop an inflammatory cellular response following FITC inoculation that is similar in magnitude and composition compared with the response in CCR2+/+ mice; and 6) CCR2−/− mice have an altered cytokine mRNA profile following FITC administration that is characterized by increased expression of GM-CSF and decreased expression of TNF-α.

Several aspects of the animal model used in this study are worthy of mention. First, the use of animals that are genetically deficient in CCR2 offers a definitive system for studying the role of CCR2 receptor signaling in the evolution of pulmonary fibrosis.
This approach avoids the use of neutralizing Abs, thereby eliminating the concerns about neutralization efficiency and complement-mediated tissue injury. Second, there is no evidence of altered collagen deposition at baseline in the CCR2\textsuperscript{−/−} and CCR2\textsuperscript{+/−} mice (Figs. 2 and 3). Third, the protection is related to CCR2 absence regardless of the fibrotic insult. Finally, the FITC model offers several advantages for the study of pulmonary fibrosis. The model is T cell independent. Histologic abnormalities are peripheral in location, patchy in nature, and nonresolving; these histologic features characterize human IPF.

CCR2 is expressed on monocytes, activated T cells, B cells, NK cells, fibroblasts, and mast cells (32–34). Expression of CCR2 is inhibited in monocytes and T cells by LPS and IFN-γ (35, 36) and up-regulated by IL-2, IL-4, IL-10, and IL-12 in T cells (35). Although MCP-1 is the principal ligand for CCR2, other ligands include MCP-2, MCP-3, MCP-4, MCP-5, and HIV Tat (37–41). However, in mice, only MCP-1, MCP-3, and MCP-5 bind CCR2. We have demonstrated that both MCP-1 and MCP-5 are elevated following a fibrotic insult with FITC. Our ELISA data in lung homogenates show that the peak expression of MCP-1 is at day 1 in wild-type mice. By RT-PCR analysis, we know that MCP-5 mRNA levels are increased at day 7 following FITC. It has been previously reported that MCP-1 levels are elevated following bleomycin treatment in rats from days 3 to 21 (42). We have extended these results to the murine model of bleomycin-induced fibrosis. Analyzing MCP-1 expression by ELISA in lung homogenates, we can demonstrate elevated levels of MCP-1 from days 1 to 14 post-bleomycin, with the peak being day 3 (data not shown). Interestingly, the kinetics of MCP-1 expression differs during FITC- and bleomycin-induced fibrosis. Although our studies do not directly address the ligand involved in CCR2-mediated effects, our data demonstrate that regulation of CCR2 expression and/or signaling have profound effects on fibrotic processes.

Our findings documenting participation of CCR2 signaling in the generation of pulmonary fibrosis are at variance with a previously reported study using anti-CCR2 neutralizing Abs (43). In those experiments, rabbit anti-CCR2 Ab was injected at days 0, 3, and 6 following bleomycin instillation (3 mg/kg). Animals were assessed for survival and hydroxyproline accumulation over a 15-day period. No effect of the CCR2 neutralization was noted on survival or fibrosis measurements. Several aspects of the model used by these investigators differ from our own. First, a much higher dose of bleomycin was used in this study compared with our experiments. The bleomycin dose resulted in ~50% mortality between days 10 and 15, whereas in our experiments the doses of bleomycin and FITC used resulted in <10% mortality at day 21. Therefore, it is possible that CCR2 plays a different role in response to high doses of injurious agents. Alternatively, the fact that CCR2 was only neutralized for the first week of injury in these experiments more likely explains the discrepancy with our work in the knockout animals that are devoid of CCR2 signaling throughout the course of the disease. Our data document that CCR2-mediated effects are prominent during the fibrotic processes rather than in the initial phases of injury. The fact that we have documented protection from pulmonary fibrosis in CCR2\textsuperscript{−/−} mice using two different model systems (bleomycin and FITC) to induce fibrosis strengthens our findings.

Our data demonstrate that CCR5\textsuperscript{−/−} mice are not protected from FITC or bleomycin-induced pulmonary fibrosis despite the fact that the CCR5 ligands MIP-1α and RANTES are induced in animal models of pulmonary fibrosis (Ref. 43, and our unpublished observations). These data demonstrate that the signaling cascade via CCR2 is not shared with the homologous CCR5 chemokine receptor. These observations highlight the importance of a specific chemokine receptor, CCR2, in the generation of a pro-fibrotic signaling cascade.

One possible explanation for the protection in CCR2\textsuperscript{−/−} mice could have been that the absence of a chemotactic receptor diminished the cellular recruitment. However, our data demonstrate that this is not the case. Despite the absence of the CCR2 receptor, there is no difference in the magnitude or composition of the inflammatory response generated to FITC compared with CCR2\textsuperscript{+/−} mice. This data can best be explained by the fact that chemotactic signals for leukocytes are redundant. Many different chemokines can recruit the same populations of leukocytes, and many chemokines can bind to shared chemokine receptors. Given this, it is not surprising that other chemotactic signals might substitute in the CCR2\textsuperscript{−/−} mice.

Although similar numbers and types of leukocytes are recruited to the lung in both CCR2\textsuperscript{+/+} and CCR2\textsuperscript{−/−} mice following FITC administration, the function of the cells is altered in CCR2\textsuperscript{−/−} mice compared with wild-type mice. These data support a role for CCR2 beyond that of a chemotactic receptor, and suggest that MCP-1 signal transduction via CCR2 plays an important role in cellular activation. The differences in cellular function are best exemplified by the alterations in the expression of TNF-α and GM-CSF following FITC inoculation in wild-type and CCR2\textsuperscript{−/−} mice.

The fact that TNF-α expression is enhanced in CCR2\textsuperscript{−/−} mice was expected given that TNF-α has been documented to be a pivotal cytokine involved in the fibrotic process. It is well established that inhibition of TNF-α signaling can diminish the fibrotic response to bleomycin (44–47); therefore, it is not surprising that this mediator is also involved in the fibrotic response to FITC. However, the fact that the CCR2\textsuperscript{−/−} mice recruited identical numbers and subsets of leukocytes to their lung in response to FITC, yet failed to activate TNF-α production, is striking.

Changes were noted in the expression of GM-CSF in FITC-treated CCR2\textsuperscript{+/+} and CCR2\textsuperscript{−/−} animals. GM-CSF is a known mitogen for alveolar epithelial cells (48), and can influence the number and activity of alveolar macrophages (Refs. 49, 50, and R. Paine, unpublished observations). Furthermore, neutralization of GM-CSF has been shown to worsen bleomycin-induced pulmonary fibrosis in both mice and rats (51, 52). Similarly, GM-CSF\textsuperscript{−/−} animals have increased bleomycin-induced pulmonary fibrosis (53). In these experiments, the increased pulmonary fibrosis in GM-CSF\textsuperscript{−/−} mice was shown to be due, in part, to diminished expression of the anti-fibrotic lipid mediator, PGE\textsubscript{2} (53). Thus, GM-CSF is a molecule known to be important in many aspects of the repair and re-epithelialization response following an acute lung injury leading to fibrosis. The elevated expression of GM-CSF in CCR2\textsuperscript{−/−} mice is evident both at days 14 and 21 post-FITC, supporting a role for GM-CSF in the re-epithelialization process.

In sum, these data suggest that CCR2 activation leads to the generation of a variety of mediators involved in the fibrotic process. We do not believe that the protection seen in the CCR2\textsuperscript{−/−} mice can be explained solely by the absence of TNF-α expression. In fact, TNF-α is a gene that is known to be induced by GM-CSF (54). Therefore, the finding of reduced TNF-α in the face of increased GM-CSF in the CCR2\textsuperscript{−/−} mice would not have been predicted. In the absence of CCR2 activation following a fibrotic insult, the signaling cascade is altered to favor the protective phenotype characterized by diminished TNF-α and increased GM-CSF. Thus, absence of CCR2 leads to a complex circumstance whereby a pro-fibrotic mediator is diminished and an anti-fibrotic mediator is enhanced.

Mediators other than those evaluated in our studies might be affected by CCR2 signaling. MCP-1 can stimulate fibroblast deposition of collagen via the up-regulation of TGF-β (55). TGF-β is
known to be up-regulated in the bleomycin model system (56, 57), and we anticipate that the same might be true for the FITC model. Thus alterations in TGF-β may contribute to the protection seen in the CCR2−/− mice. The imbalance in TNF-α production would favor the expression of pro-angiogenic CXC chemokines in the wild-type mice. Neutralization of the CXC chemokine MIP-2 has been shown to ameliorate bleomycin-induced fibrosis (58). Lastly, the fact that MCP-1 has been shown to induce/contribute to the expression of IL-4 (32, 59, 60) could suggest that in the absence of CCR2 signaling, the balance in T1/T2 cytokines is skewed to favor the production of the anti-fibrotic T1 cytokines such as IFN-γ.

The study of IPF in humans is difficult for at least three reasons.

First, the disease is idiopathic in nature in most instances, therefore, the insults that lead to the development of fibrosis are largely unknown. Second, the inability to perform serial sampling in humans makes it very difficult to study the natural history of the disease in a particular patient. Third, patients rarely present for treatment until late in the course of the disease process. Even in clinical situations such as sepsis and ARDS, where one can predict that patients will be at risk for the development of pulmonary fibrosis, it is difficult to determine which patients will actually progress to pulmonary fibrosis. CCR2−/− mice are protected from the development of pulmonary fibrosis, suggesting that therapies directed at blockade of the CCR2 receptor might provide a useful therapeutic option for clinical intervention. Additionally, CCR2 signaling does not appear to be involved in the acute inflammatory phase or the early lung injury phases of the disease, but rather, in the orchestration of the expression of pro-fibrotic mediators. This suggests that anti-CCR2 therapies could prove useful for patients presenting at later stages of the disease progression. Furthermore, the observation that CCR2−/− mice are protected from pulmonary fibrosis induced by two different agents, bleomycin and FITC, gives hope that this strategy may be widely applicable for this idiopathic human disease.

References

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