The Chemokine, CCL3, and Its Receptor, CCR1, Mediate Thoracic Radiation-Induced Pulmonary Fibrosis

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Patients receiving thoracic radiation often develop pulmonary injury and fibrosis. Currently, there are no effective measures to prevent or treat these conditions. We tested whether blockade of the chemokine, CC chemokine ligand (CCL) 3, and its receptors, CC chemokine receptor (CCR) 1 and CCR5, can prevent radiation-induced lung inflammation and fibrosis. C57BL/6J mice received thoracic radiation, and the interaction of CCL3 with CCR1 or CCR5 was blocked using genetic techniques, or by pharmacologic intervention. Lung inflammation was assessed by histochemical staining of lung tissue and by flow cytometry. Fibrosis was measured by hydroxyproline assays and collagen staining, and lung function was studied by invasive procedures. Irradiated mice lacking CCL3 or its receptor, CCR1, did not develop the lung inflammation, fibrosis, and decline in lung function seen in irradiated wild-type mice. Pharmacologic treatment of wild-type mice with a small molecule inhibitor of CCR1 also prevented lung inflammation and fibrosis. By contrast, mice lacking CCR5 were not protected from radiation-induced injury and fibrosis. The selective interaction of CCL3 with its receptor, CCR1, is critical for radiation-induced lung inflammation and fibrosis, and these conditions can be largely prevented by a small molecule inhibitor of CCR1.

Keywords: chemokine; fibrosis; radiation; lung; inflammation

Thoracic radiation is commonly used to treat cancers of the lung, esophagus, thymus, breast, and lymph nodes. Whereas radiation treatments can reduce or eliminate tumor burden, many patients also develop lung inflammation and fibrosis, which is associated with considerable morbidity and mortality. The histopathologic changes to the irradiated lung are well described, and include oxidative damage to fibroblasts and the vascular endothelium, followed by vascular congestion, inflammation, mesenchymal cell proliferation, extracellular matrix deposition, and extensive parenchymal remodeling (1). As with other forms of fibrosis, the cellular and molecular events that give rise to these conditions are poorly understood, and there are currently no effective treatments for them. Although corticosteroids are frequently administered to these patients, this approach often fails to improve the prognosis for many patients, and is associated with numerous side effects.

Radiation-induced lung injury can be divided into two phases: an early inflammatory phase, and a late fibroproliferative phase. The inflammatory phase generally occurs within 1–3 months after

CLINICAL RELEVANCE

Our research demonstrates the requirement of the chemokine, CC chemokine ligand 3, and its receptor, CC chemokine receptor (CCR) 1, in radiation-induced lung inflammation and fibrosis. A small molecule inhibitor of CCR1 prevented fibrosis in mice, and might therefore have a similar benefit in humans.

irradiation, and is characterized by alveolar cell depletion and inflammatory cell accumulation. The fibroproliferative phase occurs approximately 6 months after irradiation, and includes fibroblast proliferation, collagen accumulation, and alveolar septal thickening (2). Although the pulmonary inflammation seen during the early phase is thought to trigger the later fibroproliferative phase, the specific leukocyte subsets and molecules that link these two phases have not been identified (3, 4).

Chemokines are low-molecular weight, secreted proteins that promote directional chemotaxis of leukocyte migration through 7-TM-spanning, G protein-coupled receptors (5). Chemokines are divided into four subgroups based on the organization of the first pair of conserved cysteine motifs (6). Blocking leukocyte recruitment to inflamed tissue should, therefore, be beneficial in some inflammatory diseases (6–8). Certainly, chemokines are important mediators in the pathogenesis of lung injury in several settings (9–11). For example, binding of the chemokine, CXCL12, to its receptor, CXCR4, drives neutrophil recruitment in hypoxia-induced lung injuries (11). We and others have also found that a different chemokine, CC chemokine ligand (CCL) 3, is required for leukocyte recruitment in several models of lung inflammation, including infections with influenza virus (12) or *Aspergillus fumigatus* (13), and in graft-versus-host disease (14). Here, we show that both CCL3 and its receptor, CC chemokine receptor (CCR) 1, are required for radiation-induced lung inflammation and fibrosis, and that these conditions can be largely prevented with a small molecule inhibitor of CCR1.

MATERIALS AND METHODS

Animals

All animal studies were approved by the institutional animal care and use committee at University of North Carolina at Chapel Hill (Chapel Hill, NC). All mice were on a fully backcrossed C57BL/6 background with age-matched controls. *Ccl3*^{−/−} mice were described previously (12). *Ccr1*^{−/−} mice were purchased from Jackson Laboratories (Bar Harbor, ME), and *Ccr5*^{−/−} mice were obtained from Dr. William Kuziel (Protein Design Laboratories, Incline Village, NV).

Thoracic Irradiation

Anesthetized mice at the age of 10–12 weeks were immobilized, with a lead shield excluding all but the thoracic cavity. Animals were then

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irradiated with a single dose of 14.5 Gy from a cesium source (J. L. Shepherd and Associates, San Fernando, CA) at a dose rate of 1.65 Gy/min. LD₅₀ for C57BL/6J mice is 14.5 Gy. At this dose, survival is sufficient to permit adequate numbers of animals for long-term analyses.

Histopathology and Morphometric Analyses

Lung sections were stained with hematoxylin and eosin for routine histology, and with Masson's trichrome for collagen analysis. A total of 10 representative 5× images were analyzed in a blinded fashion for septal thickening using ImageJ software (National Institutes of Health, Bethesda, MD) software, as previously described (15).

Quantification of Lung Hydroxyproline

The right lung was acid digested and hydroxyproline content was analyzed as previously (16) (*see the online supplement for details*).

Measurements of Lung Mechanics

Anesthetized mice were mechanically ventilated on a computer-controlled small-animal ventilator (Scireq, Montreal, PQ, Canada) at 350 breaths/min, 6 ml/kg tidal volume, and positive end-expiratory pressure of 3–4 cm H₂O. Lung mechanics measurements were performed as previously described (17).

Flow Cytometric Analyses

Excised lungs were minced and digested in collagenase A for 1 hour. Red blood cells were lysed with ACK buffer, and resultant nucleated cells separated on a 20% Percoll gradient were stained with PE- or FITC-labeled monoclonal antibodies to Gr1, FoxP3, CD3, CD4, CD8, and F4/80 (Pharmingen, San Jose, CA), and analyzed on a FACScan with Cytomation software (Becton-Dickinson, Franklin Lakes, NJ).

Gene Expression Analyses

Lung RNA was extracted and quantitative real-time PCR was performed using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Data were analyzed as previously described (18).

Analyses of Cytokines

Mouse lungs were homogenized in 1 ml of protease inhibitors and centrifuged at 1,400 rpm for 15 minutes at 4°C. Collected lung supernatants were subjected to quantitative ELISA measurements using Quantikine CCL3 and Promega Transforming Growth Factor (TGF)-β1 Emax Immunoassay systems (Madison, WI).

Treatment with the CCR1 Antagonist, BX-471

Based on doses used in previous studies (19–22), the irradiated mice received twice-daily subcutaneous injections of 30 mg/kg BX-471 in 40% cyclodextrin (Sigma, St. Louis, MO) or vehicle only. Mice were killed 32 weeks for analyses after irradiation.

Lewis Lung Carcinoma Model and Irradiation

Lewis lung carcinoma cells (5×10^4) in 0.05 ml of PBS were injected subcutaneously into the anterior chest wall of wild-type (WT) and CCR1-deficient mouse. Tumor growth was assessed by using calipers for two dimensional tumor measurements (*see the online supplement for details*).

Statistical Analysis

Two-tailed Student's *t* test was performed to compare the results of hydroxyproline content, quantitative histological analyses, lung function measurement, and flow cytometric analyses. Survival analyses were performed by using Sigma Plot (San Jose, CA) with log-rank significance tests. A *P* value of less than 0.05 was considered significant.

RESULTS

CCL3 Promotes Radiation-Induced Injury

To determine whether levels of CCL3 are increased by radiation of lung tissues, we irradiated two lung-derived cell lines, MRC5 normal lung fibroblasts and SKLu-1 lung adenocarci-

noma cells, with a single dose of 5 Gy and supernatants were collected 12 hours later for CCL3 protein quantitation. Although not detectable (<1.5 pg/ml) before irradiation, CCL3 production increased significantly in both the MRC5 (5.22 ± 1.23 pg/ml) and SKLu-1 (8.12 ± 2.49 pg/ml) cell lines after irradiation. Thus, CCL3 is increased by radiation in both normal and malignant lung-derived cell lines.

We next investigated if pulmonary levels of CCL3 are also induced *in vivo* by thoracic irradiation. C57BL/6J mice were irradiated and harvested at various time points thereafter for analysis of lung CCL3 protein. By 1 week after irradiation, CCL3 levels dropped to below levels seen in untreated mice (possibly due to the death of chemokine-producing cells). By 2 weeks after irradiation, CCL3 had returned to baseline levels, and continued to rise further, reaching a peak at 4 weeks, and a second peak at 20 weeks after irradiation (Figure 1A). The mRNA expression of CCL3 showed a similar pattern in lungs of irradiated mice with peak expression at 4 and 20 weeks after irradiation (Figure 1B).

To investigate the relevance of CCL3 expression to radiation-induced lung injury, we compared radiation-induced responses in WT and CCL3-deficient mice. As expected, about half of the WT mice died by 32 weeks after irradiation (Figure 2A). However, only 16% of the *Ccl3*^{−/−} mice died after thoracic

Figure 1. Thoracic irradiation-induced CC chemokine ligand (CCL) 3 production *in vivo*. Wild-type mice were irradiated and whole lungs harvested at the indicated times for (A) CCL3 protein content by ELISA or (B) mRNA expression. Values represent ratios of increase compared with unirradiated control mice (0 wk). Values shown are the means ($\pm$ SEM). **P* < 0.05 compared with the unirradiated controls (*n* = 3–6 mice per time point).

Figure 2. Survival and fibrosis in irradiated wild-type (WT) and $Ccl3^{-/-}$ mice. (A) Survival after thoracic radiation. WT ($n = 63$) and $Ccl3^{-/-}$ mouse ($n = 31$) survival at the indicated number of weeks after irradiation. * $P < 0.001$, compared with irradiated WT mice. (B) Hydroxyproline content in the lung. Right lungs from WT and $Ccl3^{-/-}$ mice were harvested 32 weeks after radiation, and hydroxyproline assays were conducted. $Ccl3^{-/-}$ CN, unirradiated $Ccl3^{-/-}$ ($n = 9$); $Ccl3^{-/-}$ IR, irradiated $Ccl3^{-/-}$ ($n = 7$); WTCN, unirradiated WT ($n = 9$); WTIR, irradiated WT ($n = 9$). (C) Whole sections of Masson's trichrome (MT)-stained lungs of irradiated WT and $Ccl3^{-/-}$ mice. (D) MT-stained sections viewed at 20 $\times$ magnification.

irradiation. Therefore, CCL3 contributes to thoracic radiation-induced mortality. To determine if the difference in mortality between WT and CCL3-deficient mice was related to differences in fibrosis, mice were killed at 32 weeks after irradiation, and their lungs were examined for levels of collagen using a hydroxyproline assay. As expected, there was more collagen in lungs of irradiated WT mice than in their untreated counterparts (Figure 2B). Although lung collagen also increased in $Ccl3^{-/-}$ mice, this increase was significantly less than in WT mice (11 versus 27%). At 32 weeks after irradiation, a number of lung histologic changes were noted in WT mice, including inflammation, increased septal cellularity and thickness, increased collagen deposition, with disturbed architecture and organizing alveolitis (Figures 2C and 2D). In contrast, $Ccl3^{-/-}$ mice had less inflammation, no significant septal thickening, only rare and small foci of collagen deposition, and relatively intact lung architecture.

CCR1, but Not CCR5, Promotes Radiation-Induced Injury and Mortality

CCL3 binds and signals through the chemokine receptors, CCR1 and CCR5 (6). To investigate whether either of these two receptors mediates radiation-induced injury, we prepared RNA from lungs of mice at various times after irradiation. Analysis of this RNA by quantitative RT-PCR revealed that CCR1 expression was significantly increased by 4 weeks after irradiation, reached a fivefold increase over the baseline levels by 20 weeks, and remained elevated through 32 weeks (Figure 3). By contrast, CCR5 expression in the lung did not increase until displaying a slight (1.5 $\times$) increase at 12 weeks after irradiation, after which it remained elevated until Week 32. Notably, the increased CCR1 expression at 4 and 20 weeks

closely paralleled the increases seen with CCL3 expression, suggesting that CCL3 may directly lead to recruitment of CCR1-expressing cells.

Figure 3. CC chemokine receptor (CCR) 1 and CCR5 mRNA levels after thoracic irradiation. Lung total RNA was prepared from unirradiated WT mice and from irradiated WT mice at the indicated times after irradiation. Ccr1 and Ccr5 mRNA levels were assessed by quantitative PCR, and normalized to levels of glucuronidase β RNA. Values shown represent relative levels of Ccr1 and Ccr5 mRNA compared with levels in unirradiated mice ($n = 5$ –9 mice per group). Values shown are the means ($\pm$ SD). * $P < 0.05$, irradiated mice compared with unirradiated controls (0 wk).

To determine more directly the requirement of CCR1 and CCR5 in radiation-induced injury, we studied mice separately lacking these two chemokine receptors. Compared with irradiated WT mice, *Ccr1*^{-/-} mice had significantly improved survival. By contrast, there was no difference in radiation-induced mortality between WT and *Ccr5*^{-/-} mice (Figure 4A). As seen previously, radiation increased the hydroxyproline content of WT mice (~27%). Surprisingly, this increase was even greater in *Ccr5*^{-/-} mice (~50%). In contrast, but similar to what we saw in *CCL3*^{-/-} mice, hydroxyproline increases were minimal (~4%) in irradiated *Ccr1*^{-/-} mice (Figure 4B). Staining of lung sections revealed that both inflammation and collagen accumulation were also greater in *Ccr5*^{-/-} mice than in their WT counterparts (Figures 4C and 4D). In contrast, *Ccr1*^{-/-} mice

had negligible inflammation and fibrosis. Quantitative digital imaging analyses showed that, when compared with untreated controls, both the irradiated WT and *Ccr5*^{-/-} mice, but not *Ccr1*^{-/-} mice, had increased septal thickening, consistent with the hydroxyproline data (Figure 4E). Together, these findings suggest that the binding of CCL3 to CCR1 promotes radiation-induced injury, inflammation, and mortality. By contrast, binding of CCL3, or of another chemokine, to CCR5 appears to protect against radiation injury, because *Ccr5*^{-/-} mice displayed more injury than WT mice.

Decreases in lung compliance accompany and contribute to thoracic radiation-induced mortality. Accordingly, we assessed the impact of radiation on lung compliance in *Ccr1*^{-/-} mice and *Ccr5*^{-/-} mice. As expected, WT mice displayed decreased

Figure 4. Survival and fibrosis in irradiated WT, *Ccr1*^{-/-}, and *Ccr5*^{-/-} mice. (A) Survival of mice at the indicated times after irradiation. (B) Hydroxyproline content of right lungs of nonirradiated mice and irradiated mice at 32 weeks after irradiation. *CCR1*^{-/-}CN, unirradiated *CCR1*^{-/-} mice ($n = 14$); *CCR1*^{-/-}IR, irradiated *CCR1*^{-/-} mice ($n = 13$); *CCR5*^{-/-}CN, unirradiated *CCR5*^{-/-} mice ($n = 12$); *CCR5*^{-/-}IR, irradiated *CCR5*^{-/-} mice ($n = 7$); IR, irradiated; nonIR, nonirradiated control mice; WTCN, unirradiated WT mice ($n = 9$); WTIR, irradiated WT mice, $n = 9$. (C) Whole sections of MT-stained lungs of irradiated WT, *Ccr1*^{-/-} mice, and *Ccr5*^{-/-} mice. (D) MT-stained sections viewed at 20 $\times$ magnification. (E) Quantitative measurements of lung tissue abnormalities by morphometric analysis of lung sections. Ten 5 $\times$ images of each lung section were analyzed to quantify septal thickening as a measure of lung inflammation and fibrosis. Values shown are means ($\pm$ SD) of 4–8 lung sections per group. * $P < 0.05$, compared with unirradiated WT controls.

Figure 5. Leukocyte infiltration in the lung. Lungs were harvested at the indicated times after irradiation, and leukocytes prepared from them. (A–D) Antibody staining of cells for the indicated surface molecules; CD45, CD4, CD8, and F4/80. Values shown are the means ($\pm$ SEM) of three to six mice per group. * $P < 0.05$, compared with unirradiated controls of the same genotype. # $P < 0.05$, compared with irradiated WT mice at the same time point. (E) hematoxylin and eosin–stained sections of lung tissue. Arrows indicate foamy macrophages.

compliance and increased elastance after irradiation (see Figure E1 in the online supplement). Similar changes were seen in and *Ccr5*^{-/-} mice, but not in *Ccr1*^{-/-} mice. Taken together, these data suggest that CCL3, acting through CCR1, promotes radiation injury, lung inflammation, and fibrosis, with consequent impaired lung compliance and, ultimately, death.

In view of our finding that *Ccr1*^{-/-} mice, but not *Ccr5*^{-/-} mice, were protected from radiation-induced injury, we focused our subsequent studies on potential mechanisms linking CCL3 and CCR1 to fibrosis. It was unclear from our studies, thus far, if these molecules were required primarily during this late phase, or during the early inflammatory phase. To test this, we studied the progression of pulmonary inflammation over the 32 weeks between radiation and fibrosis. In lungs of irradiated WT mice, there was an initial decrease in total CD45⁺ leukocytes (Figure 5A), including CD4⁺ and CD8⁺ lymphocytes, during the first week after irradiation (Figures 5B and 5C). However, levels of each of these cell types gradually increased, and reached high levels throughout the inflammatory phase between 12 and 20 weeks after irradiation. The initial decrease in leukocytes seen

in WT mice was also seen in *Ccr1*^{-/-} mice. However, during the inflammatory phase, *Ccr1*^{-/-} mice had fewer CD45⁺ leukocytes, including CD4⁺ and CD8⁺ lymphocytes. Interestingly, macrophages, which are thought to be important during the development of fibrosis (23, 24), were significantly decreased in *Ccr1*^{-/-} mice after irradiation, but were increased in WT mice, and this difference was evident through nearly the entire 32 weeks after irradiation (Figure 5D). CCL3-deficient mice also showed decreases in CD4⁺ and CD8⁺ T cells, and macrophages compared with WT mice after thoracic radiation (S. L. Kirby, unpublished data). The decreased inflammation in *Ccr1*^{-/-} mice compared with WT mice was also evident by microscopic examination of lung tissue (Figure 5E). Of note, there were no differences in WT compared with CCR1-deficient mice in neutrophils (Gr1⁺ cells), T regulatory cells (FoxP3⁺ CD4⁺), or B cells (CD19⁺ CD20⁺). Taken together, these findings suggest that binding of CCL3 to CCR1 during the first few weeks after irradiation contributes to radiation-induced lung inflammation.

Several reports have shown that cytokines produced by T helper (Th) 2 lymphocytes are associated with fibrosis (25, 26).

Figure 6. T helper (Th) 1 and Th2 cytokine gene expression after radiation. Lung total RNA was prepared from unirradiated WT mice and from irradiated WT mice at the indicated times after irradiation. mRNA levels were assessed by quantitative PCR and normalized to levels of glucuronidase β RNA. Values shown represent relative levels compared with levels in unirradiated mice. (A) IL-4; (B) IL-13; (C) IFN- γ . Values shown are the means ($\pm$ SD) of six to nine mice per group. * $P < 0.05$, compared with unirradiated controls of the same genotype.

Accordingly, we studied levels of the Th2 cytokines, IL-4 and IL-13, and compared them to levels of the Th1 cytokine, IFN- γ (Figures 6A–6C). Both IL-4 and IL-13 were increased in lungs of irradiated WT mice, with increases in IL-4 preceding those of

IL-13 (Figures 6A and 6B). However, no significant increases in these cytokines were seen in *Ccr1*^{-/-} mice. Instead, the latter animals had increased levels of the Th1 cytokine, IFN- γ (Figure 6C). This finding demonstrates that CCR1 is required for the development of a Th2 environment in the lung after thoracic radiation, and suggests that CCR1-mediated recruitment of Th2 cells might contribute to radiation-induced fibrosis.

Because pulmonary fibrosis is associated with TGF- β 1 production (27), we compared levels of this cytokine in WT, *Ccl3*^{-/-}, and *Ccr1*^{-/-} animals at 32 weeks after irradiation. TGF- β 1 levels were significantly increased in irradiated WT mice, but not in irradiated *Ccl3*^{-/-} or *Ccr1*^{-/-} mice (Figure E2).

The CCR1 Inhibitor, BX-471, Attenuates Radiation-Induced Lung Injury

The protection from the lung inflammation and fibrosis seen in *Ccl3*^{-/-} mice and *Ccr1*^{-/-} mice suggested that pharmacologic inhibition of CCL3 binding to CCR1 might diminish or prevent radiation-induced lung injury. One of the most extensively studied small molecule inhibitors of CCR1 is BX-471, which can ameliorate inflammation in several rodent disease models (21, 22, 28, 29). Accordingly, we tested the ability of BX-471 to protect WT mice from radiation-induced injury and death. We began administration of this inhibitor at 3 weeks after irradiation (14.5 Gy), because it was shortly after this time that both leukocytes and CCR1 expression begin to increase in our model (Figure 3). The inhibitor, BX-471, was given twice daily for 4 weeks. Control irradiated mice received vehicle (40% cyclodextrin) only for 4 weeks. This short course of BX-471 conferred nearly complete protection from radiation-induced lung fibrosis, as shown by hydroxyproline assays and by histologic analyses of lung tissue (Figures 7A and 7B). The survival at 32 weeks was significantly improved by treatment with the inhibitor (96% [BX-471 treated] versus 67% [vehicle treated]). The inhibitor also prevented the decline in lung function seen in irradiated mice that received vehicle only (Figure E3). Similar cohorts of irradiated WT mice treated for 8 weeks with BX-471 (Week 3 to Week 11 after irradiation) showed similar, but not better, results. Thus, short-term treatment with BX-471 was sufficient to prevent radiation-induced fibrosis.

The ability of BX-471 to prevent radiation-induced fibrosis and decline in lung function in our model suggests that this compound might also prevent these complications in humans. Patients receive thoracic radiation for tumors. A number of tumors express CCR1 (30, 31), and CCR1 has been implicated, either directly or through host defense, as a promoter of tumor growth or metastases (32–35). Thus, it is important to examine whether BX-471 may alter tumor growth. To investigate this, we injected Lewis lung carcinoma cells into C57BL/6 mice that also received BX-471 (or vehicle only in control mice). The transplanted tumor cells grew to an equivalent extent in untreated WT mice as in BX-471-treated WT mice or vehicle-treated *Ccr1*^{-/-} mice (Figure E4). Therefore, we are encouraged that BX-471 does not accelerate growth of these tumor cell lines.

DISCUSSION

The development of pulmonary fibrosis is associated with prior inflammation, but a direct and causal relationship between these two events has not been established. In the model used here, genetic deletion of CCL3 or its receptor, CCR1, protected against both inflammation and fibrosis, suggesting that these two events were causally linked. However, with regard to fibrosis, these experiments did not identify a temporal requirement of CCL3 and CCR1 during only the inflammatory phase, during only the fibroproliferative phase, or during both phases of the

Figure 7. Pulmonary fibrosis in irradiated WT mice treated with BX471. (A) Collagen levels in the right lung as determined by measurements of hydroxyproline at 32 weeks after irradiation. CNTL, unirradiated and untreated, number of mice per group ($n = 12$); IR+BX471, irradiated and treated with daily subcutaneous injection of BX-471 at 30 mg/Kg of body weight from Week 3 to Week 7 after irradiation ($n = 19$). IR+vehicle, irradiated and treated with daily subcutaneous injection of vehicle (40% cyclo-dextrin) from Week 3 to Week 7 after irradiation ($n = 16$). (B) Whole sections of MT-stained lungs of irradiated mice treated with BX471 or vehicle. (C) Representative MT-stained lung sections of mice treated with BX471 or vehicle.

disease. Inhibitors are particularly valuable in this regard, because they can be selectively administered during one phase, but not the other. Our finding, that inhibitor-mediated blockade of CCR1 during a 4-week period during the onset of the inflammatory phase is sufficient to prevent both the lung inflammation and fibrosis in WT mice, provides strong evidence that the inflammatory phase is required for development of the subsequent fibroproliferative phase.

We found that the deficiencies of CCL3 receptors, CCR1 and CCR5, had different impacts on radiation lung injury. Whereas *Ccr1*^{-/-} mice had relatively little disease compared with WT mice, *Ccr5*^{-/-} mice had more severe disease than WT mice. The selective requirement of CCR1 for fibrosis in this model is consistent with the previous findings that anti-CCL3 antibodies, as well as anti-CCR1 antibodies, reduce bleomycin-induced inflammation and collagen deposition (36–38), that *Ccr1*^{-/-} mice are protected from hepatic fibrosis (39) and renal fibrosis (29), and that BX-471 protects mice against acute pancreatitis-associated lung injury by modulating neutrophil recruitment (40). However, the exacerbated disease seen in *Ccr5*^{-/-} mice was not anticipated. The dramatic difference in disease severity between these two strains might derive from the different leukocyte subsets that are recruited by these two

chemokine receptors. Whereas CCR1 is reported to promote Th2 cell recruitment (41), CCR5 is expressed on Th1 cells and promotes Th1-type immune responses (42). The ability of Th2 cytokines to activate fibroblasts and promote extracellular matrix deposition and remodeling (43) suggests that CCR1 can enhance fibrosis by recruiting Th2 cells to the lung. By contrast, the Th1 cytokine, IFN- γ , has antifibrotic effects, possibly by inhibiting the function of myofibroblasts (44, 45). Thus, CCR5 might inhibit the development of fibrosis by recruiting Th1 cells to the lung. This explanation is supported by our finding that Th2 cytokines began to increase at about 4 weeks after radiation in WT mice, but not in *Ccr1*^{-/-} mice. An alternative explanation is that CCR5 promotes the recruitment T regulatory cells, as it is proposed to do in other settings (46). The severity of disease in *Ccl3*^{-/-} mice was intermediate between that of *Ccr1*^{-/-} mice and of *Ccr5*^{-/-} or WT mice, suggesting that, whereas CCL3 recruits effector cells through CCR1, it also recruits protective cells through CCR5. Disease in *Ccl5*^{-/-} mice was also intermediate between WT and *Ccr1*^{-/-} mice (S.L.K., unpublished data). Additional studies will be required to characterize more extensively the phenotype and function of each of these cell types, and the impact of CCR1 and CCR5 on their recruitment.

The data presented here suggest that radiation-induced fibrosis is mediated by the binding of CCL3 to its receptor, CCR1, whereas binding of this chemokine to a different receptor, CCR5, protects against fibrosis. Importantly, selective blockade of CCR1 with the antagonist, BX-471, effectively protects WT mice from the lung injury and fibrosis that is otherwise seen in these animals after radiation. These novel findings suggest that blockade of the CCL3/CCR1 signaling pathway might also protect human patients from thoracic radiation-induced pulmonary fibrosis.

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