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Essential Roles of the CC Chemokine Ligand 3-CC Chemokine Receptor 5 Axis in Bleomycin-Induced Pulmonary Fibrosis through Regulation of Macrophage and Fibrocyte Infiltration

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We investigated the pathogenic roles of CC chemokine ligand (CCL)3 and its receptors, CC chemokine receptor (CCR)1 and CCR5, in bleomycin (BLM)-induced pulmonary fibrosis (PF). An intratracheal injection of BLM into wild-type (WT) mice caused a massive infiltration of granulocytes and macrophages, followed by the development of diffuse PF with fibrocyte accumulation. Intrapulmonary CCL3 expression was enhanced rapidly and remained at elevated levels until PF developed. Moreover, CCL3 protein was detected mainly in infiltrating granulocytes and macrophages, whereas transforming growth factor-β1 protein was detected in macrophages and myofibroblasts. Compared with WT mice, collagen accumulation was reduced in CCL3$^{-/-}$ and CCR5$^{-/-}$ but not CCR1$^{-/-}$ mice. Moreover, the BLM-induced increases in intrapulmonary macrophage and fibrocyte numbers were attenuated in CCL3$^{-/-}$ and CCR5$^{-/-}$ but not CCR1$^{-/-}$ mice, although BLM increased bone marrow (BM) fibrocyte number to a similar extent in these strains. BM transplantation from CCR5$^{-/-}$ to WT, but not that from WT to CCR5$^{-/-}$ mice, recapitulated the phenotypes in CCR5$^{-/-}$ mice. Furthermore, CCR5$^{+/-}$ mice exhibited a significant reduction in BLM-induced fibrotic changes. These results demonstrated that locally produced CCL3 was involved in BLM-induced recruitment of BM-derived macrophages and fibrocytes, main producers of transforming growth factor-β1, and subsequent development of PF by interacting mainly with CCR5. ***(Am J Pathol 2007, 170:843–854; DOI: 10.2353/ajpath.2007.051213)***

Pulmonary fibrosis (PF) is a chronic lung disease characterized histologically by diffuse interstitial inflammation and fibrosis.[1,2] PF frequently develops because of well-defined entities, such as irradiation injury, oxygen toxicity pneumonitis, and scleroderma, but approximately half of the cases are idiopathic. Idiopathic pulmonary fibrosis (IPF) is a progressive and irreversible chronic lung disease, and most patients experience severe hypoxemia and cyanosis in the advanced stages. Given the absence of proven therapies for IPF, current clinical management is primarily supportive, and lung transplantation is sometimes tried to cure the situation.[1,2]

Bleomycin (BLM), a copper-chelating peptide that can cleave DNA, is widely used as an anti-tumor agent for various types of malignancies, including squamous carcinomas and lymphomas, because BLM administration seldom causes myelosuppression, a common adverse effect of other various types of anti-tumor agents.[3] However, BLM treatment frequently causes pulmonary injury, and irreversible PF sometimes ensues as its severe form.[3] Because pathological changes in BLM-induced PF resemble those in IPF, a BLM-induced PF model is used to elucidate the molecular and cellular pathogenesis of IPF. On BLM administration, mice develop acute alveolitis and interstitial inflammation, characterized by the sequential infiltration of neutrophils, macrophages, and lymphocytes. The infiltration of inflammatory cells is postulated to lead to myofibroblast hyperplasia and dis-

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ordered collagen deposition.[4] Recently, in addition to these inflammatory cells, several independent groups proposed that fibrocytes expressing both leukocyte (CD45, CD13) and mesenchymal antigens (collagen I, fibronectin) have crucial roles in fibrosis, including BLM-induced PF and renal fibrosis.[5–12]

Chemokines can recruit and activate a selected type(s) of inflammatory cells, and some can mobilize even fibrocytes.[7–10,12] BLM treatment can augment the intrapulmonary expression of various chemokines including macrophage inflammatory protein-1α/CC chemokine ligand (CCL)3, a member of CC chemokines.[13–20] CCL3 exhibits chemotactic and activating effects on neutrophils, macrophages, lymphocytes, and immature dendritic cells.[21–23] Moreover, CC chemokine receptor (CCR)1 and CCR5, a specific receptor for CCL3,[24,25] are expressed by fibrocytes.[7,9] Indeed, passive immunization of BLM-challenged mice with anti-CCL3 antibodies attenuated fibrotic changes,[13] implying CCL3 as a mediator responsible for BLM-induced fibrosis. However, its precise roles have not been determined yet, particularly with reference to fibrocytes. Moreover, it remains to be investigated how CCR1 and CCR5 are used by a single ligand, CCL3, in BLM-induced PF.

Here, we investigated the pathophysiological roles of the CCL3 axis in BLM-induced PF by using mice deficient in each gene. We demonstrated that the genetic ablation of CCL3 markedly attenuated PF induced by BLM administration. Moreover, homozygous and to a lesser degree, heterozygous deficiency in CCR5, ameliorated significantly fibrotic changes in the lungs, whereas CCR1 deficiency has minimal effects on BLM-induced PF. Furthermore, the recruitment of fibrocytes in both $CCL3^{-/-}$ and $CCR5^{-/-}$ mice was significantly reduced compared with WT and $CCR1^{-/-}$ mice. Finally, bone marrow (BM) transplantation from WT to $CCR5^{-/-}$ mice restored the susceptibility to BLM, whereas WT mice receiving $CCR5^{-/-}$ BM were protected from BLM-induced PF. Thus, CCL3 used CCR5 but not CCR1 to recruit and activate BM-derived cells including fibrocytes in BLM-induced PF.

Materials and Methods

Reagents and Antibodies (Abs)

BLM was purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-mouse CCR5 polyclonal antibodies (pAbs) were prepared as described previously.[26] The following monoclonal antibodies (mAbs) and pAbs were commercially obtained: rat anti-mouse F4/80 mAb (Dainippon Pharmaceutical Co., Osaka, Japan); rat anti-mouse Ly-6G mAb, fluorescein isothiocyanate (FITC)-labeled rat anti-mouse CXC chemokines receptor (CXCR)4 mAb, peridinin chlorophyll-a protein (PerCP)-labeled rat anti-mouse CD45 mAb, and FITC-labeled rat anti-mouse CD45 mAb (BD Pharmingen, San Jose, CA); goat anti-mouse CCL3 pAbs (R&D Systems, Inc., Minneapolis, MN); rabbit anti-transforming growth factor (TGF)-β1 pAbs and goat anti-CCR5 pAbs (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-α-smooth muscle actin (α-SMA) mAb (Boehringer Mannheim GmbH, Mannheim, Germany); Alexa Fluor 488-labeled hamster anti-CCR5 (Biolegend Inc., San Diego, CA); Cy3-conjugated donkey anti-rat IgG pAbs, FITC-conjugated donkey anti-goat IgG pAbs, and FITC-conjugated donkey anti-rabbit IgG pAbs (Jackson Immunoresearch Laboratories, West Grove, PA); and rabbit anti-type I collagen (Col I) pAbs (Chemicon International, Temecula, CA).[8,11,12]

Mice

Pathogen-free 8-week-old male C57BL/6 mice were obtained from Sankyo Laboratories (Tokyo, Japan) and designated as wild-type (WT) mice. Homozygous CCL3-deficient ($CCL3^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME). CCR1-deficient ($CCR1^{-/-}$) mice were a generous gift from Drs. P.M. Murphy and J.-L. Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).[27] CCR5-deficient ($CCR5^{-/-}$) mice were generated as previously described.[28] All these mice were backcrossed to C57BL/6 mice for 8 to 10 generations. In some experiments, $CCR5^{-/-}$ mice were mated with C57BL/6 mice to generate heterozygous CCR5-deficient ($CCR5^{+/-}$) mice. All mice were kept under the specific pathogen-free conditions at the Animal Research Center of Kanazawa University (Kanazawa, Japan). Age- and sex-matched mice were used for the experiments. All animal experiments in this study complied with the Guidelines for the Care and Use of Laboratory Animals on the Takara-Machi Campus of Kanazawa University.

BLM-Induced Lung Injury

After mice were anesthetized with an intraperitoneal injection of pentobarbital (50 μg/g weight), a cervical midline incision was made, and the trachea was exposed. Thereafter, BLM (0.075 U) in 50 μl of sterile saline was intratracheally administered by the use of a syringe with a 26-guage needle. At the indicated time intervals after BLM administration, the mice were sacrificed by an overdose of diethylether, and both lungs were removed for subsequent analyses.

Generation of BM Chimera Mice

The following BM chimeric mice were prepared: male $CCR5^{-/-}$ BM→female WT mice, male WT BM→female $CCR5^{-/-}$ mice, male WT BM→female WT mice, and male $CCR5^{-/-}$ BM→female $CCR5^{-/-}$ mice. BM cells were collected from the femurs of donor mice by aspiration and flushing. Recipient mice were irradiated to 15 Gy using an RX-650 irradiator (Faxitron X-ray Inc., Wheeling, IL). Then, the animals received intravenously 5×10^6 BM cells from donor mice in a volume of 200-μl sterile phosphate-buffered saline (PBS)(−) under the anesthesia. Thereafter, the mice were housed in sterilized microisolator cages and were fed normal chow and autoclaved hyperchlorinated water for 60 days. To verify successful engraftment and reconstitution of the BM in the transplanted mice, genomic DNA was isolated

Table 1. Sequences of the Primers Used for RT-PCR

Transcript	Sequence	Annealing temperature (°C)	Cycle	Product size (bp)
CCL3	(F) 5′-GCCCTTGCTGTTCTTCTCTGT-3′ (R) 5′-GGCATTCAGTTCCAGGTCAGT-3′	58	35	258
CCL4	(F) 5′-GCTCTGTGCAAACCTAACCC-3′ (R) 5′-CTGAGGAGGCCTCTCCTGAAGT-3′	55	32	360
CCL5	(F) 5′-CATATGGCTCGGACACCACT-3′ (R) 5′-ACACACTTGGCGGTTCCTTC-3′	60	30	146
TGF-β1	(F) 5′-CGGGGCGACCTGGGCACCATCCATGAC-3′ (R) 5′-CTGCTCCACCTTGGGCTTGCGACCCAC-3′	60	35	405
Col I	(F) 5′-GGGCAAGACAGTCATCGAAT-3′ (R) 5′-TTGGTTTTTGGTCACGTTCA-3′	60	35	339
	(F) 5′-ACCACCAAGACCTCCCGCCTG-3′* (R) 5′-AAGGTAAAAACTAAGTTTGAA-3′**	60	35	286
β-Actin	(F) 5′-TTCTACAATGAGCTGCGTGTGGC-3′ (R) 5′-CTCATAGCTCTTCTCCAGGGAGGA-3′	60	28	456

(F), Forward primer; (R), reverse primer.
* and **Primers were used for nested PCR to prepare RNA probes for *in situ* hybridization.

from peripheral blood and tail tissues of each chimeric mouse 30 days after BMT with a NucleoSpin tissue kit (Macherey-Nagel, Duren, Germany). Then, we performed polymerase chain reaction (PCR) to detect the *Sry* gene contained in the Y chromosome (forward primer, 5′-TTGCCTCAACAAAA-3′; reverse primer, 5′-AAACTGCTGCTTCTGCTGGT-3′). The amplified PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. After durable BM engraftment was confirmed, mice were treated with BLM as described above.

Histopathological Analyses

The lung tissues were fixed in 10% formalin buffered with PBS (pH 7.2) and embedded in paraffin. Six-μm-thick sections were made and stained with hematoxylin and eosin or Masson's trichrome for the detection of collagen deposition. Histopathological changes were evaluated by an examiner without prior knowledge on the experimental procedures. Immunohistochemical analyses were also performed using anti-Ly-6G, anti-F4/80, anti-CCL3, or TGF-β1 Abs, as described previously.[29] The numbers of infiltrating granulocytes or macrophages were enumerated on 10 randomly chosen visual fields at $\times$200 magnification, and the average of the 10 selected microscopic fields was calculated. All measurements were performed by an examiner without previous knowledge on the experimental procedures. The incubation of isotype-matched control Ab did not give rise to any positive reaction, indicating the specificities of each primary Ab (data not shown). As described previously,[29,30] preadsorption of anti-CCL3 or anti-TGF-β1 Abs with an excess amount of each recombinant protein abolished the positive signals, further indicating the specificity of the reaction (data not shown).

Double-Color or Triple-Color Immunofluorescence Analysis

A double-color immunofluorescence analysis was conducted to identify the types of CCL3- and TGF-β1-expressing cells in the lung, as described previously.[30] A triple-color immunofluorescence analysis was also performed in the combination of anti-CD45, anti-Col I, and anti-CCR5 Abs. Thereafter, the sections were observed under a fluorescence microscopy. In triple-color analysis, we titrated the concentrations of anti-Col I to determine the concentration that did not give rise to a positive staining in extracellular matrix.

Determination of Hydroxyproline (Hyp) Contents

At 21 days after BLM administration, lung tissues were removed, and the contents of Hyp, a major component of collagen, were determined as previously described.[29] The data were expressed as the amount (μg) per lung.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Five μg of total RNA extracted from lung samples was reverse-transcribed as described previously. The resultant cDNAs were amplified together with *Taq* polymerase (Takara Shuzo, Kyoto, Japan) using the specific sets of primers with the optimal cycles consisting of 94°C for 30 seconds, the optimal annealing temperature for 30 seconds, and 72°C for 1 minute, followed by incubation at 72°C for 5 minutes (Table 1). The amplified PCR products were fractionated on a 2% agarose gel along with a constant amount of a standard DNA and visualized by ethidium bromide staining.[29,30] The band intensities were measured using NIH Image Analysis software (version 1.63), and the ratios to β-actin were calculated on the assumption that the ratios of untreated animals are 1.0.

Flow Cytometric Analysis

Mononuclear cells were isolated from lungs, BM, and peripheral blood, as described previously.[8] Nonspecific binding was blocked by incubating with 25 μg/ml of Fc block (BD Biosciences Pharmingen, Piscataway, NJ) for 15 minutes at 4°C. The cells were stained with FITC-

labeled anti-CXCR4 or Alexa Fluor 488-labeled anti-CCR5 and PerCP-labeled anti-CD45. After an extensive washing with PBS (−), the cells were permeabilized using cytofix/cytoperm (BD Biosciences Pharmingen) and stained with anti-Col I pAbs followed by the incubation with PE-conjugated goat anti-rabbit Ig (Molecular Probes Inc., Eugene, OR) as previously described.[8,11,12] After being washed twice with PBS (−), the cells were fixed in 2% paraformaldehyde. Control staining was performed by using isotype-matched mAbs for CD45, CXCR4, or CCR5. For immunostaining with anti-collagen I pAb, normal rabbit IgG was used as a negative control. A flow cytometric analysis with FITC-labeled anti-CD45 and Via-Probe7-AAD (BD Pharmingen) indicated that dead cells were consistently less than 0.5% in the cells gated with anti-CD45 mAb. Moreover, the cells were stained with anti-Col I, anti-CXCR4, and CCR5 for the evaluation of CCR5-positive fibrocytes co-expressing CXCR4. The stained cells were analyzed on a FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA), and the obtained data were analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA).

In Situ *Hybridization Combined with Immunofluorescence Analysis on CD45$^+$ Cells from the Whole Lungs and BM*

At 14 days after BLM administration, mononuclear cells were isolated from lungs and BMs and cultured for 10 to 14 days, as described previously.[9] The trypsinized cells were stained with anti-CD45 mAb coupled to magnetic beads (Miltenyi Biotech, Auburn, CA). The cells were then sorted by LS-positive selection columns using a SuperMACS apparatus (Miltenyi Biotech) according to the manufacturer's instructions. After being washed extensively, CD45$^+$ cells, which were retained on the column, were removed from the magnetic field and were used for *in situ* hybridization combined with immunofluorescence analysis.[31] Nested RT-PCR products of collagen I were obtained using the pair of primers with the addition of T7- and Sp6-RNA polymerase promoter to the 5′ end of each sense and anti-sense primer of collagen I, respectively (Table 1). Digoxigenin-labeled sense and anti-sense probes were obtained by using DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. The sense probe was used as a negative control. After the resultant CD45$^+$ cells were incubated with anti-sense probe of collagen I, the bound probe was detected using rhodamine-conjugated anti-digoxigenin Abs. Thereafter, anti-CXCR4 Abs were further applied to the cells, followed by the incubation of FITC-labeled secondary Abs. Images were observed under a fluorescent microscopy and digitally merged.

Enzyme-Linked Immunosorbent Assay (ELISA)

Lung samples were homogenized with PBS containing Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and centrifuged at 5000 × *g* for 10 minutes. Supernatants were used to determine CCL3, TGF-β, and CXC chemokine ligand (CXCL)12 levels with commercial ELISA kits (R&D Systems), according to the manufacturer's instructions. The detection limits in each method were as follows: CCL3 > 1.5 pg/ml, TGF-β1 > 7 pg/ml, and CXCL12 > 40 pg/ml. Total protein in the supernatant was measured with a commercial kit (bicinchoninic acid protein assay kit; Pierce, Rockford, IL). The data were expressed as the target molecule (pg) per total protein (mg) for each sample.

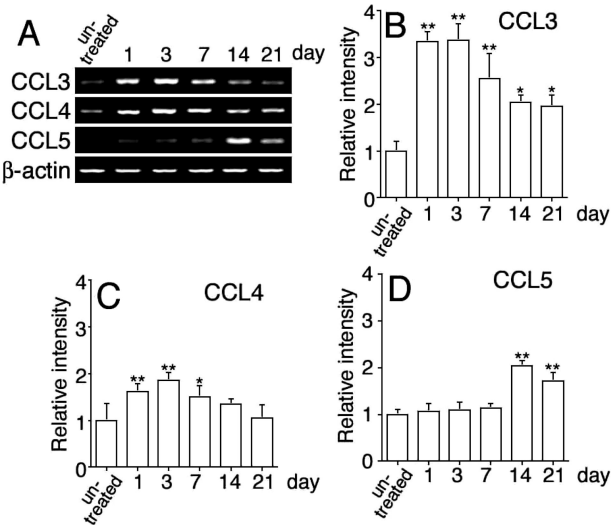

Figure 1. CC chemokine expression in the lungs of WT mice after BLM treatment. **A–D:** RT-PCR was performed on total RNAs extracted from the lungs at the indicated time intervals after BLM treatment as described in Materials and Methods. Representative results from six independent experiments are shown in **A**. The ratios of CCL3 (**B**), CCL4 (**C**), and CCL5 (**D**) to β-actin were calculated. Each value represents mean ± SEM ($n = 6$). $^*P < 0.05$; $^{**}P < 0.01$, versus untreated WT lungs.

Statistical Analysis

The means and SEMs were calculated for all parameters determined in this study. Statistical significance was evaluated by using one-way analysis of variance with posthoc testing with the Scheffé's *F* multiple comparisons test or Mann-Whitney's *U*-test. $P < 0.05$ was accepted as statistically significant.

Results

Chemokine Expression in Lung after BLM Treatment

CCL3 mRNA expression was increased nearly threefold as early as 1 day and remained elevated until 14 days after BLM injection (Figure 1, A and B). In contrast, CCL4 mRNA expression was marginally increased and returned to a basal level at 14 days after the injection and CCL5 mRNA expression was enhanced only later than 14 days after the injection (Figure 1, A and D). These observations suggest that CCL3 was a major chemokine expressed in lungs after BLM treatment among these chemokines. Moreover, in-

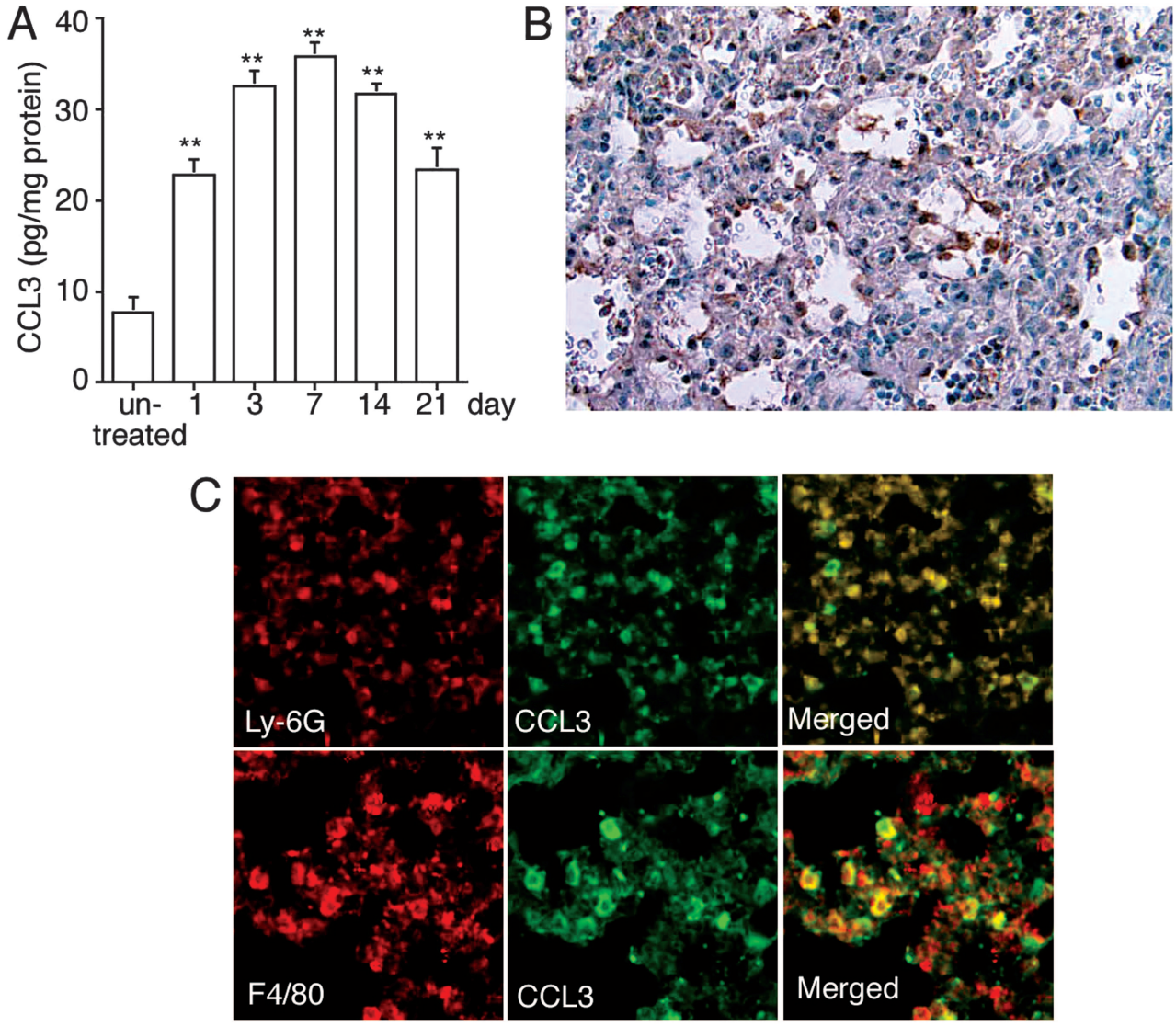

Figure 2. Intrapulmonary CCL3 protein expression after BLM treatment. **A:** Intrapulmonary CCL3 contents were determined by ELISA and are shown here. Each value represents mean ± SEM ($n = 6$). $^{**}P < 0.01$, versus untreated WT lungs. **B:** Immunohistochemical detection of CCL3 protein in the lungs of WT mice at 7 days after BLM treatment. A representative result from six independent experiments is shown here. **C:** A double-color immunofluorescence analysis of CCL3-expressing cells in the lungs of WT mice at 7 days after BLM treatment. Representative results from six independent experiments are shown here. The fluorescent images were digitally merged (right column). Original magnifications, ×200 (**B**).

trapulmonary CCL3 protein contents started to be increased 1 day after BLM treatment and reached a peak at 14 days (Figure 2A). Furthermore, CCL3 protein was immunohistochemically detected in polymorphonuclear or mononuclear leukocytes recruited into the alveolar spaces after BLM treatment (Figure 2B). A double-color immunofluorescence analysis detected CCL3 protein in Ly-6G- and F4/80-positive cells (Figure 2C) but not CD3-positive cells (data not shown) after BLM treatment, suggesting that granulocytes and macrophages were main producers of CCL3.

Attenuated PF in CCR5$^{-/-}$ and CCL3$^{-/-}$ Mice after BLM Treatment

To evaluate the contribution of CCL3 and its receptors to BLM-induced lung injury, the mice deficient in CCL3, CCR1, and CCR5 were injected with BLM, along with WT mice. Before BLM challenge, no apparent histological differences were observed in the lungs among these mice (data not shown). By 3 days after administration, the lungs of WT mice exhibited marked hemorrhages and congestion with infiltration of inflammatory cells predominantly consisting of Ly-6G-positive polymorphonuclear cells (Figures 3 and 4). Later than 7 days, F4/80-positive mononuclear inflammatory cells predominated in infiltrating cells, with a marked thickening of the alveolar septa (Figures 3 and 4). Concomitantly, fibrotic changes and fibroblast proliferation become apparent, starting from the subpleural areas and extending to the central areas. At 21 days after BLM administration, Masson's trichrome staining revealed a massive blue coloration in the lungs from WT mice

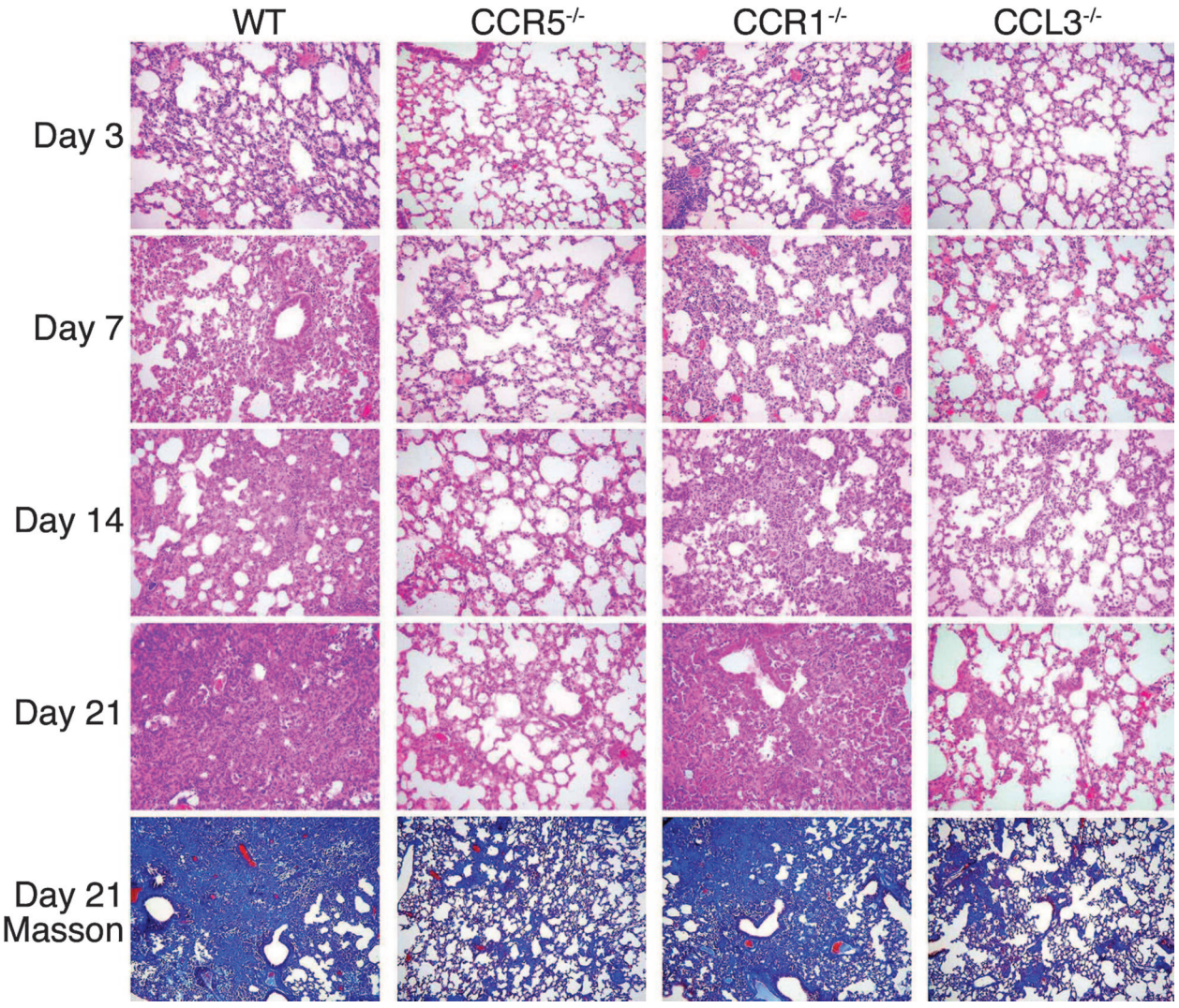

Figure 3. Histopathological observations on lungs from WT, CCR5$^{-/-}$, CCR1$^{-/-}$, and CCL3$^{-/-}$ mice after BLM treatment. Representative results from six animals at each time point are shown. Original magnifications: ×100 (H&E stain); ×40 (Masson's stain).

(Figure 2), indicating a dense collagen deposition. CCR1$^{-/-}$ mice also exhibited similar histopathological changes as WT mice after BLM treatment (Figure 3). In contrast, these pathological changes were remarkably attenuated in CCL3$^{-/-}$ and CCR5$^{-/-}$ mice with a marked reduction in Ly-6G-positive granulocyte and F4/80-positive macrophage infiltration, compared with WT mice (Figures 3 and 4). Moreover, we evaluated intrapulmonary Hyp contents, an index of collagen deposition. Before BLM challenge, there was no significant difference in intrapulmonary Hyp contents among these strains (data not shown). Consistent with histopathological analysis, intrapulmonary Hyp contents, an index of collagen deposition, were increased approximately threefold in WT and CCR1$^{-/-}$ mice 21 days after BLM administration, whereas the increases in Hyp contents were negligible in CCL3$^{-/-}$ and CCR5$^{-/-}$ mice (Figure 5). These observations would indicate that the CCL3-CCR5 but not the CCL3-CCR1 axis was involved in BLM-induced lung injury. Moreover, the intrapulmonary Hyp contents in CCR5$^{+/-}$ mice were intermediate between BLM-treated WT and CCR5$^{-/-}$ mice 21 days after BLM treatment (data not shown), suggesting that CCR5 gene has dose effects on BLM-induced PF.

Contribution of BM Cells to BLM-Induced PF

Evidence is accumulating to indicate the crucial contribution of BM cells to PF.[8–10] To evaluate the roles of BM-derived cells to BLM-induced PF, we made several BM chimeric mice. Although radiation can induce pneumonitis including fibrosis, the untreated BM chimeric mice did not exhibit a significant increase in intrapulmonary Hyp (Figure 6), indicating that radiation alone cannot induce PF under the present conditions. BLM injection increased Hyp contents markedly and to a similar level in WT BM→CCR5$^{-/-}$ mice and WT BM→WT mice as WT mice (Figure 6). Although the intrapulmonary Hyp contents were slightly increased in CCR5$^{-/-}$ BM→WT mice and CCR5$^{-/-}$ BM→CCR5$^{-/-}$ mice, the extent of fibrotic

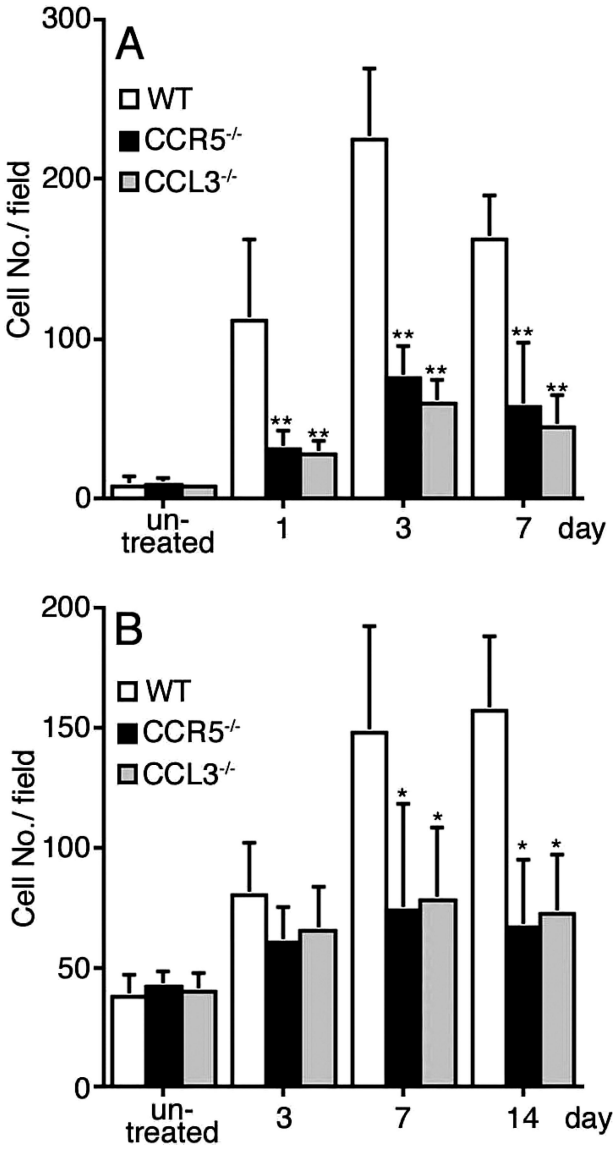

Figure 4. The numbers of granulocytes and macrophages in the lung tissue after BLM treatment. Lung tissues were obtained from WT, CCL3$^{-/-}$, and CCR5$^{-/-}$ mice at the indicated time intervals after BLM treatment and were immunostained with anti-Ly6G and anti-F4/80 antibodies to determine the numbers of granulocytes (**A**) and macrophages (**B**), respectively. The cell numbers were counted per microscopic field at ×200 magnification. Each value represents mean ± SEM ($n = 6$). *$P < 0.05$; **$P < 0.01$, versus WT mice at the same time points.

changes was significantly reduced, compared with mice transplanted with WT BM. Thus, BM-derived cells were mainly responsible for BLM-induced PF.

CCR5 Deficiency Impaired Fibrocyte Migration into the BLM-Injected Lungs

The potential contribution of BM-derived cells to BLM-induced fibrosis prompted us to examine the roles of BM-derived fibrocytes, which are presumed to be involved in PF.[8,9] In line with previous reports,[8] we detected specific collagen I mRNA in some of CXCR4$^+$ cells among CD45$^+$ cells isolated from whole lungs

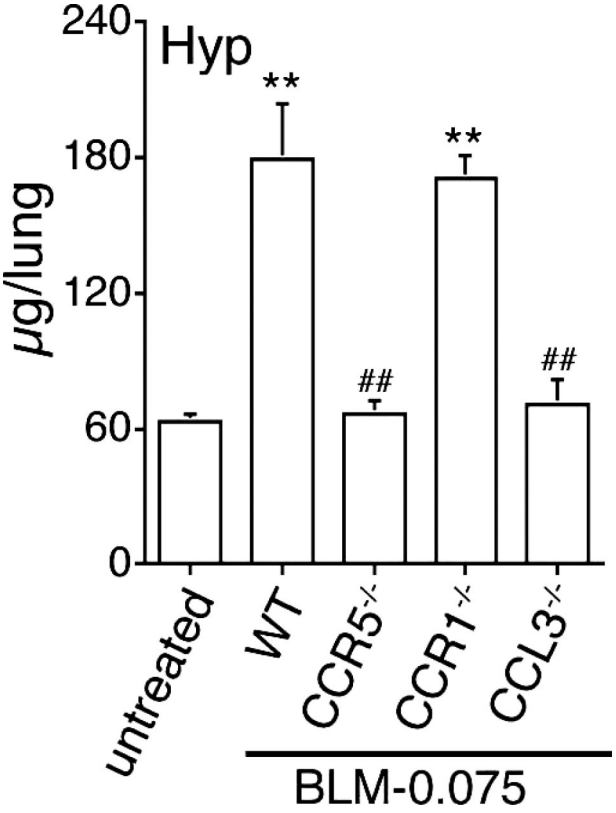

Figure 5. Hyp contents in the lung at 21 days after BLM treatment. Hyp contents were determined as an indicator of collagen contents. Each value represents mean ± SEM ($n = 6$). **$P < 0.01$, versus untreated WT lungs. ##$P < 0.01$, versus BLM-treated WT lungs.

(Figure 7A) and BMs (data not shown). Sense probes showed no positive signals, indicating the specificity of the reaction (data not shown). Before BLM treatment, there was no difference in the number of the intrapulmonary fibrocytes, defined as CD45$^+$CoII$^+$CXCR4$^+$ cells, among untreated WT mice and mice deficient in CCR5, CCR1, and CCL3. BLM increased the number of the intrapulmonary fibrocytes to similar extents in WT and CCR1$^{-/-}$ mice 14 days after the injection (Figure 7, B and C), but the increase was marginal in CCR5$^{-/-}$ or CCL3$^{-/-}$ mice, and the increment was intermediate in CCR5$^{+/-}$ mice (Figure 7C). Thus, the intrapulmonary fibrocyte numbers were well correlated with BLM-induced increases in intrapulmonary Hyp contents among these strains. Moreover, similar numbers of fibrocytes were present in BM and, to a lesser degree, peripheral blood among these four untreated strains (Figure 7D). An intratracheal injection of BLM increased the fibrocyte numbers in BM but not peripheral blood, to similar extents among these four strains 14 days after the injection (Figure 7D), indicating that BM is a source of fibrocytes. Moreover, CCR5 was detected on CD45$^+$Col I$^+$ cells (Figure 7E), consistent with the previous reports.[7,9] Moreover, CXCR4 was coexpressed by ~90% of CCR5$^+$ fibrocytes, which were recruited into the lungs of WT mice after BLM treatment (Figure 7F). In the next series, we analyzed the number of CD45$^+$CoII$^+$CCR5$^+$ fibrocytes in WT

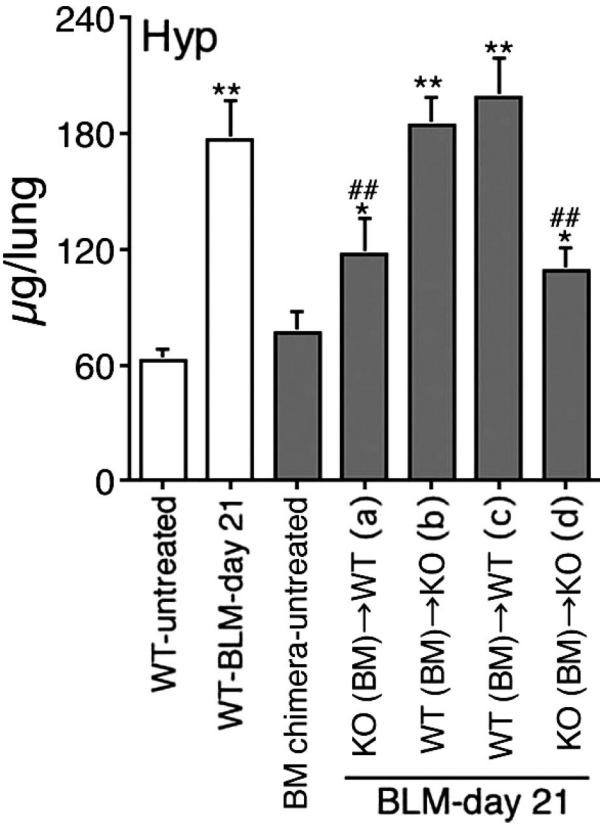

Figure 6. The effects of BM transplantation on BLM-induced PF. Recipient female mice were transplanted with BM cells from CCR5$^{-/-}$ or WT male donors as described in Materials and Methods. BM chimera mice were injected with BLM 60 days after BM transplantation. Intrapulmonary Hyp contents were determined as an indicator of collagen contents 21 days after BLM treatment as described in Materials and Methods. There was no difference in Hyp contents among all four BM chimera mice before BLM challenge. Each value represents mean ± SEM ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$, versus untreated WT lungs. $^{\#\#}P < 0.01$, versus BLM-treated WT mice.

and CCL3$^{-/-}$ mice. Although there was no difference in intrapulmonary CCR5^{+} fibrocyte numbers between untreated WT and CCL3$^{-/-}$ mice (data not shown), BLM induced less CCR5^{+} fibrocyte recruitment into the lungs in CCL3$^{-/-}$ mice, compared with WT mice (Figure 8, A–C). Thus, the CCL3-CCR5 axis can regulate the migration of fibrocytes into the lungs from the BM, and eventually PF was induced by BLM treatment.

Attenuated Expression of Intrapulmonary CXCL12 Expression in CCR5$^{-/-}$ and CCL3$^{-/-}$ Mice

CXCL12, a single ligand for CXCR4, is also presumed to be involved in the migration of fibrocytes from the BM.[8] In WT mice, intrapulmonary CXCL12 protein contents started to be increased 1 day and reached a peak 7 days after BLM challenge. Moreover, CXCL12 protein contents remained at fourfold higher levels than untreated ones, even at 21 days after BLM treatment (Figure 9). In contrast, both CCR5$^{-/-}$ and CCL3$^{-/-}$ mice exhibited a remarkable attenuation in intrapulmonary CXCL12 protein levels, compared with WT mice.

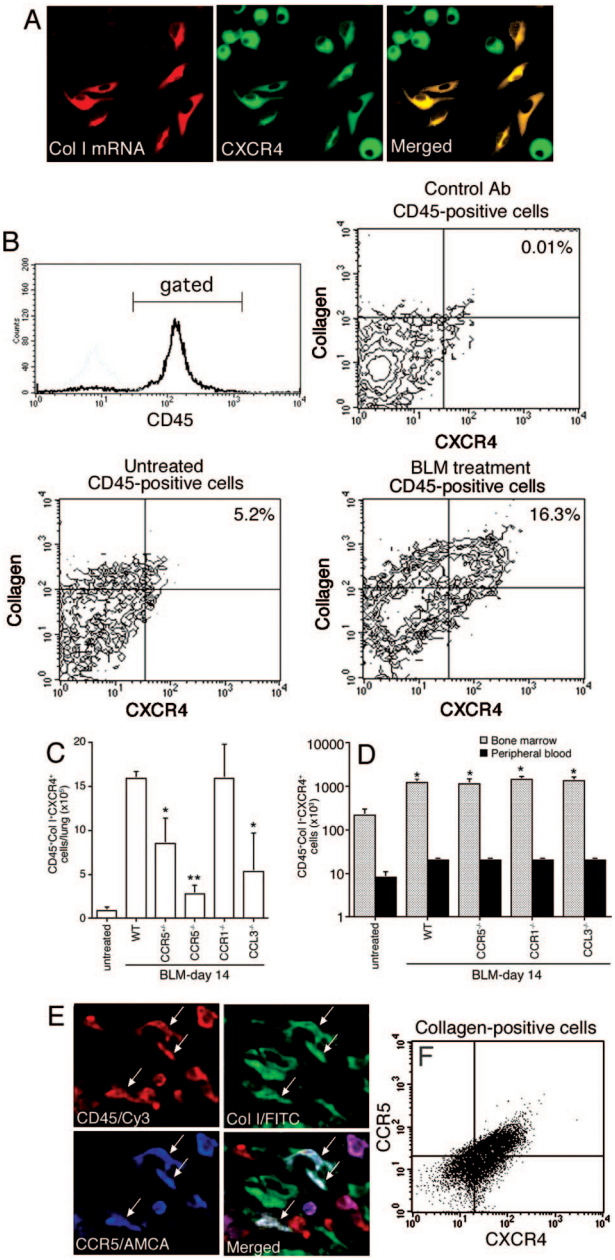

Figure 7. A: Detection of collagen I mRNA in CXCR4^{+} CD45^{+} cells. CD45^{+} cells, which were isolated from the whole lung were processed to *in situ* hybridization and immunofluorescence analysis, to detect collagen I mRNA and CXCR4 expression, respectively, as described in Materials and Methods. Representative results from six independent experiments are shown here. Similar results were obtained when BM-derived CD45^{+} cells were used. Original magnifications, ×400. **B:** Flow cytometric analysis on CD45^{+}CXCR4^{+}Col I^{+} fibrocytes in the lungs, BM, and peripheral blood at 14 days after BLM challenge. Single-cell suspensions were isolated from the BM, peripheral blood, and lungs, and stained with the combination of anti-CD45, anti-CXCR4, and anti-Col I Abs, followed by a flow cytometric analysis, as described in Materials and Methods. Representative results on lung single cell suspensions from six individual WT mice are shown here with a contour plotting. Normal rabbit IgG was used as a negative control to gate collagen-positive signals (**B**, top left). **C:** The numbers of fibrocytes were calculated on lungs. Each value represents mean ± SEM ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$, versus BLM-treated WT mice. **D:** The numbers of fibrocytes were calculated on BM and peripheral blood. Each value represents mean ± SEM ($n = 6$). $^{*}P < 0.05$, versus untreated mice. **E:** A triple-color immunofluorescence analysis of CCR5-expressing cells in the lungs of WT mice at 14 days after BLM challenge. CD45^{+}Col I^{+} fibrocytes expressed CCR5 (**arrows**). Representative results from six independent experiments are shown here. Original magnifications, ×400. **F:** Flow cytometric analysis on the portion of CCR5-positive fibrocytes co-expressing CXCR4. Representative results are shown here.

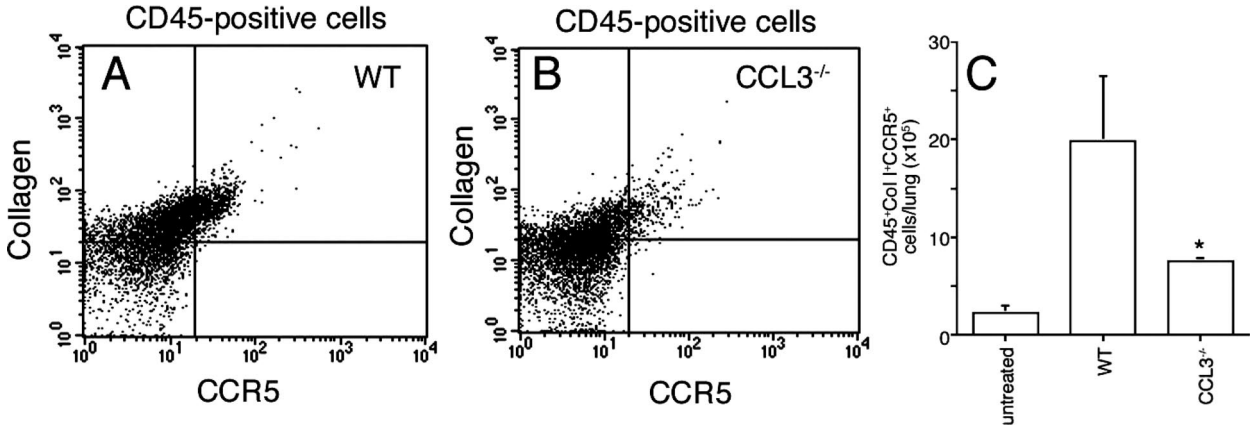

Figure 8. **A** and **B:** Flow cytometric analysis on CCR5$^+$ fibrocytes in the lungs of WT and CCL3$^{-/-}$ mice at 14 days after BLM treatment. Single-cell suspensions were isolated from lungs and stained with the combination of anti-CD45, anti-CCR5, and anti-Col I Abs, followed by a flow cytometric analysis. Representative results from six independent experiments are shown in **A** (WT) and **B** (CCL3$^{-/-}$). **C:** The numbers of intrapulmonary CCR5$^+$ fibrocytes were calculated, and are shown here. Each value represents mean ± SEM ($n = 6$). $^*P < 0.05$, WT versus CCL3$^{-/-}$ mice.

Thus, the lack of the CCL3-CCR5 axis might impair fibrocyte recruitment directly and indirectly by reducing CXCL12 expression.

TGF-β1 Expression after BLM Treatment

We finally examined the intrapulmonary expression of TGF-β1, a potent stimulating factor for fibroblast proliferation and collagen production.[32,33] Among untreated WT, CCR5$^{-/-}$, and CCL3$^{-/-}$ mice, there were no significant differences in TGF-β1 expression at mRNA and protein levels (Figure 10, A and B). BLM treatment markedly enhanced TGF-β1 expression at mRNA and protein levels in WT mice, whereas the enhanced TGF-β1 expression was significantly reduced in both CCR5$^{-/-}$ and CCL3$^{-/-}$ mice, compared with WT mice (Figure 10, A and B). Moreover, immunohistochemical and double-color immunofluorescence analyses demonstrated that F4/80- or α-SMA-positive cells were cellular sources of TGF-β1 in WT mice after BLM treatment (Figure 10, C and D). Thus, the absence of CCL3 or CCR5 may reduce the intrapulmonary migration of fibrocytes, a precursor of fibroblasts, and F4/80-positive macrophages, cell populations that produced TGF-β1, a factor responsible for fibrotic changes.

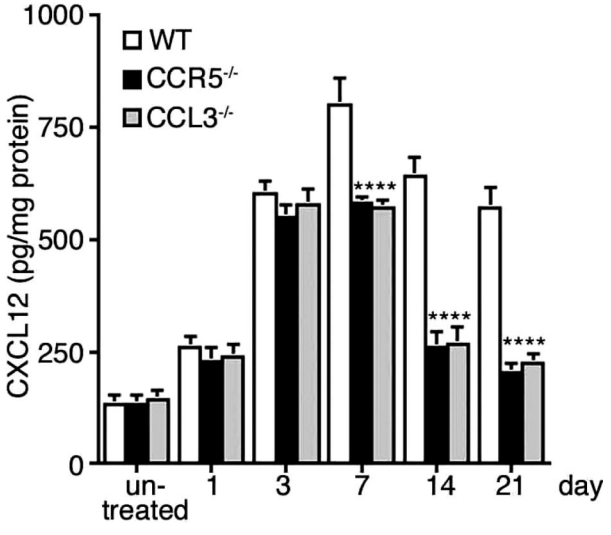

Figure 9. Intrapulmonary CXCL12 protein contents after BLM treatment. Intrapulmonary CXCL12 contents in WT, CCR5$^{-/-}$, and CCL3$^{-/-}$ mice were determined by ELISA and are shown here. Each value represents mean ± SEM ($n = 6$). $^{**}P < 0.01$ versus WT mice at the same time points.

Discussion

Some chemokines show redundancy in terms of utilization of receptors. CCL3 utilizes two distinct receptors, CCR1 and CCR5, as its specific receptors. Simultaneously, these two receptors are used by CC chemokines other than CCL3.[24,25,34] *In vitro* studies have revealed that there are slight differences in expression patterns between CCR1 and CCR5. CCR1 is expressed by macrophages, granulocytes, NK cells, T cells, and immature dendritic cells,[35–37] whereas CCR5 is expressed by T cells, B cells, immature and mature dendritic cells, and macrophages.[38–41] In the present study, the deficiency of CCL3 and CCR5 but not CCR1 gene remarkably attenuated BLM-induced fibrotic changes in lungs, implying that the CCL3-CCR5 axis was more essential in the development of BLM-induced PF than the CCL3-CCR1 axis. Moreover, the observations on BM-chimeric mice have revealed the essential roles of BM-derived cells.

Our observations on CCL3$^{-/-}$ mice were consistent with the previous observation that the passive immunization of BLM-challenged mice with anti-CCL3 Ab attenuated markedly fibrotic changes.[13] In contrast, our observations on CCR1-deficient mice are seemingly contradictory to the previous report demonstrating that anti-CCR1 Ab improved BLM-induced intrapulmonary fibrotic changes.[18] In that study, a much higher dose of BLM was used and caused subacute massive lung injury, resulting in 45% mortality 14 days after BLM challenge, whereas we did not see any mortality with our present dose. Moreover, granulocyte infiltration persisted longer in their model than ours. Because granulocytes express CCR1 but not CCR5, anti-CCR1 Ab might act on the infiltrating granulocytes, thereby reducing BLM-induced PF in their model. On the other hand, because some CCR1-positive cells in lungs also expressed

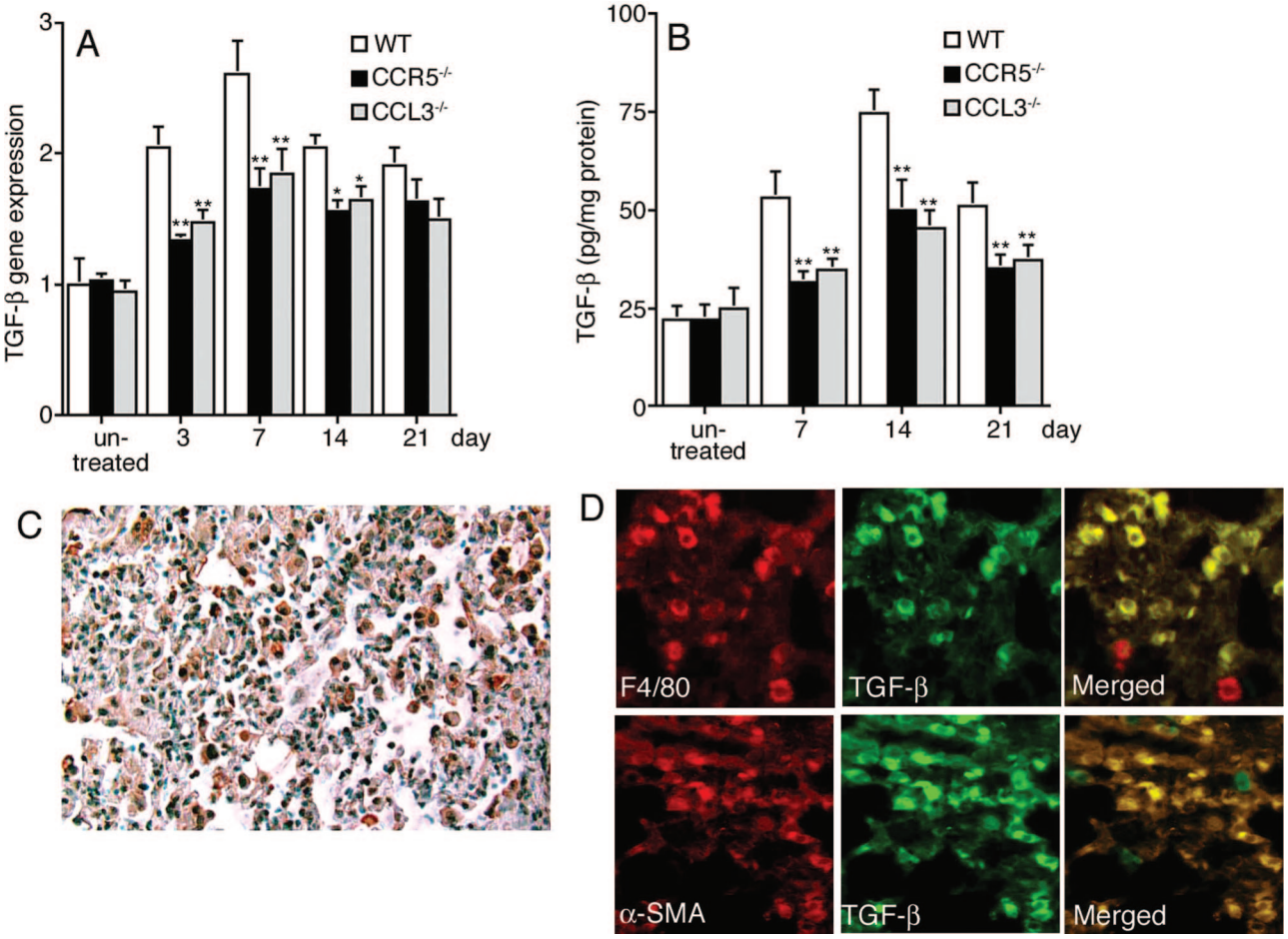

Figure 10. TGF-β1 expression in lungs after BLM treatment. **A:** RT-PCR analysis for TGF-β1 was performed. The ratios of TGF-β1 to β-actin of WT, CCR5$^{-/-}$, and CCL3$^{-/-}$ mice were determined and are shown here. Each value represents mean ± SEM ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ versus WT mice at the same time points. **B:** Intrapulmonary TGF-β1 protein contents were determined by ELISA as described in Materials and Methods. Each value represents mean ± SEM ($n = 6$). $^{**}P < 0.01$ versus WT mice at the same time points. **C:** Immunohistochemical analysis for TGF-β protein was performed. A representative result at 14 days after BLM treatment is shown here. **D:** A double-color immunofluorescence analysis of TGF-β1-expressing cells. Lungs were obtained from WT mice at 14 days after BLM treatment. The sections were stained with the combination of anti-α-SMA (Cy3) and anti-TGF-β1 (FITC) and observed under fluorescent microscopy. The fluorescent images were digitally merged (right column). Representative results from six independent experiments are shown here. Original magnifications: ×200 (**C**); ×400 (**D**).

CCR5 simultaneously (our unpublished data), anti-CCR1 Ab treatment may impair the functions of these double-positive cells as genetic CCR5 deficiency may do, thereby reducing lung fibrotic changes.

Moore et al[19] reported that CCR5$^{-/-}$ mice on a C57BL/6 × 129 F2 genetic background developed lung fibrosis to a similar extent as WT mice when administered with 0.025 U of BLM. However, we observed the same dose did cause little pathological changes in the lungs of WT mice on a C57BL/6 background (our unpublished data). Similar differences were also found in the overall magnitude of the fibrotic response to FITC between C57BL/6 and C57BL/6 × 129 F2 mice.[9] Thus, the genetic background may account for this seeming discrepancy.

Fibrotic changes are a hallmark of the BLM-induced lung injury and are characterized histologically by fibroblast hyperplasia and increased collagen deposition. Several lines of evidence have revealed that BLM can augment the intrapulmonary expression of TGF-β1, one of the most potent fibrogenic factors[32,33] and that BLM-induced PF was ameliorated by counteracting the signal pathways of TGF-β1.[42,43] These observations imply TGF-β1 as one of the main mediators responsible for BLM-induced PF. We also demonstrated that BLM-induced enhancement of TGF-β1 expression was significantly attenuated in the lungs of CCR5$^{-/-}$ and CCL3$^{-/-}$ mice, compared with WT mice. We observed that CCR5-positive cells started to infiltrate to the lungs 3 days after BLM injection and that most CCR5-positive cells were F4/80-positive macrophages (our unpublished data). Given that F4/80-positive macrophages were one of the main producers of TGF-β1, the CCL3-CCR5 axis can regulate the trafficking of macrophages, thereby contributing to development of BLM-induced PF.

In addition to F4/80-positive macrophages, α-SMA-positive myofibroblasts were positive for TGF-β1 in our model. Because TGF-β1 can induce fibroblasts to produce collagen,[32,33] TGF-β1 may act in an autocrine manner. Several lines of evidence suggest that some but not all fibroblasts are derived from a circulating population of cells, termed fibrocytes, which express both leukocyte (CD45, CD34, CD13) and mesenchymal markers (type I collagen, fibronectin).[5–12,44–46] Of interest is that fibrocytes can exhibit a chemotactic

response to several chemokines including CXCL12, CCL2, and CCL21.[7–9,12] Indeed, the neutralization of CXCL12 or the lack of CCR2 (a specific receptor for CCL2) reduced the recruitment of fibrocytes into the lungs and eventually reduced intrapulmonary collagen deposition after BLM or FITC challenge.[8,9] These observations would indicate that chemokine-mediated trafficking of fibrocytes to the lung may essentially be involved in the pathogenesis of PF.

Two independent groups detected CCR5 mRNA and protein and CCR1 mRNA in fibrocytes,[7,9] but the pathophysiological relevance has not been determined yet. Hence, we examined the fibrocyte numbers in BM, peripheral blood, and lungs among CCL3$^{-/-}$, CCR1$^{-/-}$, and CCR5$^{-/-}$ mice before and after BLM injection. BLM treatment increased fibrocyte numbers in BM to similar extents among these strains, but the increments in peripheral blood were negligible. However, the intrapulmonary fibrocyte numbers were increased to a similar extent in WT and CCR1$^{-/-}$ but not CCL3$^{-/-}$ and CCR5$^{-/-}$ mice. Moreover, CCL3$^{-/-}$ mice exhibited impaired CCR5^{+} fibrocyte recruitment compared with WT mice. These observations suggest the involvement of the CCL3-CCR5 axis in fibrocyte migration from BM but not fibrocyte expansion in BM. Moreover, fibrocyte numbers in lungs were correlated with intrapulmonary collagen deposition after BLM treatment, among these strains. Given that CCL3 was abundantly expressed in lungs from the early time points after BLM treatment, locally produced CCL3 may interact with CCR5 on fibrocytes to induce their migration from BM to lungs. Migrated fibrocytes may eventually differentiate to myofibroblasts, one of main sources of fibrogenic factor, TGF-β1, thereby causing fibrotic changes.[9]

Intrapulmonary CXCL12 protein was also significantly reduced in CCL3$^{-/-}$ and CCR5$^{-/-}$ mice, compared with WT mice. A double-color immunofluorescence analysis detected CXC12 protein mainly in F4/80-positive macrophages (our unpublished data). Given a potent capacity of CXCL12 to induce fibrocyte migration, the absence of CCL3 and CCR5 attenuated BLM-induced intrapulmonary fibrocyte accumulation directly and/or indirectly by reducing the migration of macrophages, a main source of CXCL12.

We have demonstrated that both homozygous and heterozygous CCR5-deficient mice were less prone to develop a major adverse effect of BLM lung fibrosis. In humans, 32-bp deletion allele in *ccr5* gene is prevalent among Caucasians, with a frequency of 0.092.[47–49] This deletion results in a frame-shift, with a premature stop codon. Thus, homozygous persons do not express functional CCR5 protein at all, similarly to CCR5$^{-/-}$ mice. Both homozygous and hemizygous individuals do not exhibit any apparent health problems but are resistant to human immunodeficiency virus (HIV)-1 infection because CCR5 is a co-receptor for T-tropic HIV.[50–53] Thus, it is probable that the polymorphisms of CCR5 among individuals may determine the sensitivities to BLM. If so, typing of human CCR5 polymorphisms may help to individualize BLM treatment for malignancies.

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