Complex Regulation of Pulmonary Inflammation and Fibrosis by CCL18

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Elevated pulmonary levels of CCL18 have been associated with influx of T lymphocytes, collagen accumulation, and a decline in lung function in pulmonary fibrosis patients. We previously reported that overexpression of CCL18 in mouse lungs triggers selective infiltration of T lymphocytes and moderate lymphocyte-dependent collagen accumulation. We hypothesized that in combination with bleomycin injury, overexpression of CCL18 will worsen the severity of lung inflammation and fibrosis. Mice were infected with a replication-deficient adenovirus encoding CCL18 and then instilled with bleomycin; control mice were challenged with either CCL18 overexpression or bleomycin. Additive effects of CCL18 overexpression and bleomycin injury were observed on pulmonary inflammation, particularly on T-cell infiltration, and increased levels of tumor necrosis factor- α , interferon- γ , matrix metalloproteinase (MMP)-2, and MMP-9. Despite the additive effect on inflammation, CCL18 overexpression unexpectedly attenuated the bleomycin-induced collagen accumulation. Pulmonary levels of active transforming growth factor- β 1 mirrored the changes in collagen levels. Depletion of T cells with antilymphocyte serum or pharmacological inhibition of MMPs with GM6001 abrogated accumulation of collagen and increases in the levels of tumor necrosis factor- α , interferon- γ , and active transforming growth factor- β 1. Thus, CCL18-stimulated T-lymphocytic infiltration is by itself mildly profibrotic to a healthy lung, whereas it partially protects against lung fibrosis in an inflammatory profibrotic pulmonary milieu. (*Am J Pathol* 2007; 171:428–437; DOI: 10.2353/ajpath.2007.061167)

rophages and bronchoalveolar lavage (BAL) fluid, respectively, in patients with scleroderma lung disease,¹ a condition characterized by the accumulation of T cells in the lungs and by pulmonary fibrosis.² Increases in CCL18 have also been reported in the lungs of patients with other pulmonary diseases characterized by T-cell involvement and collagen deposition, such as hypersensitivity pneumonitis and idiopathic pulmonary fibrosis,^{3,4} pulmonary sarcoidosis,⁵ and allergic asthma.^{6,7}

CCL18, which is also known as pulmonary and activation-regulated chemokine (PARC), macrophage inflammatory protein 4 (MIP-4), alternative macrophage activation-associated CC chemokine 1 (AMAC-1), dendritic cell-derived chemokine 1 (DC-CK1), and small secreted cytokine A 18 (SCYA-18), is constitutively expressed at high levels in the lungs^{3,8–10} and is selectively chemotactic for T cells.¹¹ We recently reported^{12–14} and others have recently confirmed⁴ that CCL18 in high concentrations (300 to 1000 ng/ml) acts directly on cultured primary pulmonary fibroblasts, activates intracellular signaling, and stimulates collagen production in a time- and dose-dependent manner. Macrophages produce CCL18 on so-called alternative activation in a Th2 environment^{8,15–17} and directly stimulate collagen production in fibroblasts.¹⁸ *In vivo*, CCL18 may promote pulmonary fibrosis when expressed at much lower concentrations (300 pg/ml) by attracting T cells to the lungs.¹¹

The T cells constitute a relatively minor population in a normal lung; this population expands numerically and undergoes phenotypic changes in association with lung inflammation and fibrosis.^{2,19–22} Although not all pulmonary fibrotic processes are T-lymphocyte-dependent,^{23–25} previous studies suggested that T lymphocytes do contribute to regulation of fibrosis in the lung in

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We recently reported that levels of a CC chemokine CCL18 mRNA and protein are increased in alveolar mac-

human disease^{1,2,19–21} and in animal models of pulmonary fibrosis.^{22,26,27} In contrast to pulmonary T lymphocytes, macrophages are by far the most abundant cell type in the lungs, normally constituting more than 85% of bronchoalveolar lavage cells.² Although the percentage of macrophages declines during lung inflammation due to influx of T cells and other inflammatory cells, macrophages undergo phenotypic changes associated with inflammation and fibrosis¹ and establish a vicious circle of pulmonary fibrosis by further up-regulating CCL18 expression.⁴

We have recently developed a CCL18 overexpression animal model that resembles human pulmonary fibrotic disease; it manifests in pulmonary T-lymphocytic infiltration, transforming growth factor (TGF)- β activation, and T-cell-dependent collagen accumulation.¹¹ However, in humans with pulmonary fibrosis, the elevation of pulmonary levels of CCL18 occurs in the context of pulmonary inflammation and fibrosis that may affect the outcome of CCL18 expression in the lungs. Considering that both CCL18 expression¹¹ and inflammation²⁸ are profibrotic, we hypothesized that the combined action of CCL18 overexpression and bleomycin-induced lung injury would cause profound additive or synergistic fibrotic lung damage. We report here that unexpectedly, CCL18 overexpression is partially protective against bleomycin-induced injury. We also begin addressing the mechanisms of this phenomenon by following the changes in the levels of the factors that known to be involved in the regulation of fibrosis, including matrix metalloproteinases MMP-2 and MMP-9 and cytokines TGF- β 1, interleukin (IL)-13, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ .^{29–33} We conclude that the CCL18-attracted pulmonary T lymphocytes act profibrotically in otherwise healthy lungs but partially antifibrotically in the presence of a second profibrotic injury (bleomycin). The implication of this observation is that a therapeutic elimination of T lymphocytes from the inflamed lungs may have a counterintuitive deleterious effect. Furthermore, there might be potential for further enhancing the antifibrotic regulation in the lungs by therapeutically manipulating the local pulmonary milieu and/or the phenotypes of infiltrating T lymphocytes.

Materials and Methods

Experimental Animal Models

Ten- to 12-week-old C57BL/6 female mice weighing 18 to 21 g were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in sterile microisolator cages with sterile rodent feed and acidified water. Daily maintenance of mice was performed in the Baltimore VA Medical Center Research Animal Facility that is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were treated in accordance with a research protocol that has been approved by the University of Maryland Institutional Animal Care and Use Committee. Mice

were weighed daily using a calibrated scale. Recombinant adenoviral vectors AdV-CCL18 and AdV-NULL were constructed, validated, and used as previously reported.¹¹ Intratracheal instillation of 50 μ l of bleomycin solution (Sigma-Aldrich, St. Louis, MO) or phosphate-buffered saline (PBS) was performed in a fashion similar to instillation of adenoviruses.¹¹

To address the possibility that the effects of CCL18 on collagen production *in vivo* may depend on the presence and severity of the inflammatory milieu, intratracheal instillation of AdV-CCL18 or, as controls, of AdV-NULL or PBS was performed (day 0). Selected animals were sacrificed to confirm the expected CCL18 expression dynamics as previously reported (see Figure 1 in Ref. 11). At the peak of CCL18 production (day 7), intratracheal instillation of bleomycin was performed to induce lung inflammation and fibrosis; alternatively, PBS was instilled as control. As a result of these two instillations, four groups of mice were formed, as described below. The control group was instilled first with AdV-NULL or PBS and then with PBS again; we have previously reported that mice instilled with AdV-NULL or PBS were not phenotypically or histologically different beyond three days following instillation.¹¹ The CCL18 alone group overexpressed CCL18 as a result of AdV-CCL18 instillation; the second instillation in this group was with PBS. The bleomycin alone (BLM) group received the first instillation of either AdV-NULL or PBS, and the second instillation of either 0.01 or 0.03 U of bleomycin. These doses of bleomycin were selected as insufficient to achieve a plateauing effect on lung inflammation and fibrosis, based on the results of preliminary experiments in which titrated doses (0.005 to 0.1 U per mouse) were instilled (data not shown). Finally, the combined CCL18 overexpression and bleomycin injury group (CCL18 + BLM) received AdV-CCL18 (first instillation) and bleomycin (second instillation). On day 21 following the first instillation (day 14 after the second instillation), mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. ELISA assays of lung homogenates were used to confirm the expected decline of CCL18 levels by day 21 following AdV-CCL18 instillation¹¹; such decline was independent of the nature of the agent used for the second instillation (bleomycin or PBS).

For depletion of lymphocytes, some of the animals were first injected with antilymphocyte serum (Accurate, Westbury, NY), on days -4, -2, and 0 relevant to the first intratracheal instillation, and the decrease in the amount of lymphocytes to <5% of the initial levels was confirmed by flow cytometry, as described.¹¹ In some other cases, mice were treated with a broad-spectrum MMP inhibitor GM6001 (Chemicon, Temecula, CA) intraperitoneally at 2 mg/mouse daily for the last 5 days before euthanasia or with anti-MMP-9-neutralizing antibody (Calbiochem, San Diego, CA) intraperitoneally at 60 μ g/mouse on days 14 and 18 after the first instillation.

Histological Examination of the Lungs, Bronchoalveolar Lavage, and Flow Cytometry

Immediately postmortem, the lungs were rapidly dissected free of extraneous tissues and filled with either formalin-free fixative (Anatech, Battle Creek, MI) for subsequent hematoxylin and eosin staining or with 1:1 mixture of PBS and TissueTek OCT compound (Sakura, Torrance, CA) for subsequent immunohistochemical analyses. Cryostat sections, stainings, controls, and image analyses were performed as described.¹¹ For BAL, the animals were euthanized, and lung lavage was performed immediately postmortem through an 18-gauge blunt-end needle secured in the trachea as described.¹¹ Differential cell counts in BAL samples were performed after the staining of cytospin preparations with a Protocol Hema 3 staining set (Fisher, Kalamazoo, MI) by at least two technicians who were blinded to the identity of the samples. The flow cytometric analyses of BAL cells were performed after staining with directly labeled antibodies (BD PharMingen, San Diego, CA) or corresponding isotype controls as described.¹¹

Determination of Hydroxyproline Content, Enzyme-Linked Immunosorbent Assay (ELISA) Analyses, and Zymographic Analyses of Lung Homogenates

Pulmonary levels of hydroxyproline were measured as surrogate of total collagen, as described.¹¹ In brief, the snap-frozen lungs were crushed under liquid nitrogen, thawed in 0.5 ml of PBS containing a protease inhibitor cocktail (Sigma), and further homogenized in a glass homogenizer. The solid tissue was separated by centrifugation; the supernatant was diluted twofold with the ELISA sample buffer, and used for ELISA analyses of total and active TGF- β 1, IL-13, TNF- α , IFN- γ , monocyte chemoattractant protein-1 (MCP-1) (CCL2), total (pro- and active) MMP-2, and pro-MMP-9 (all kits purchased from R&D Systems, Minneapolis, MN). Total protein was measured using a Bio-Rad assay (Hercules, CA). The solid tissue was hydrolyzed in 5 N NaOH at 120°C for 30 minutes in an autoclave. The mixture was then reacted with chloramine T and Ehrlich's reagent to produce a chromophore, which was quantified by spectrophotometry at 550 nm. A second aliquot of the original lung homogenate was used for colorimetric detection and quantification for total protein content using the Bio-Rad assay. For zymographic analyses, lungs were homogenized in 50 mmol/L Tris-HCl buffer containing 1 mmol/L monothiolglycerol, and the solid tissue was separated by centrifugation. The supernatants were normalized for total protein and loaded onto Novex 10% zymogram gels containing 0.1% gelatin (Invitrogen, Carlsbad, CA). After electrophoretic separation, gels were renatured, developed at 37°C overnight, and stained with Colloidal Blue stain (Invitrogen) following manufacturer's recommendations.

In Vitro Chemotaxis Assays

Chemotaxis assays were used as described¹¹ to determine whether CCL18 selectively attracts regulatory T cells. In brief, human T cells purified from peripheral blood mononuclear cell as described¹¹ were seeded in triplicates in the upper chamber using Costar Transwell inserts (3- μ m pore size; Costar, Cambridge, MA) and incubated, with or without rhCCL18 in the lower chamber, at 37°C for 4 hours. The cells that migrated into the lower chamber, as well as the cells that remained in the upper chamber, were analyzed for expression of cell surface CD4, CD25, and intracellular FoxP3 by flow cytometry.

Statistical Analyses

Data are reported as the mean $\pm$ SD. Differences between groups were evaluated with Student's two-tailed unequal variance *t*-test and Mann-Whitney *U*-test. *P* values less than 0.05 were considered statistically significant.

Results

Pulmonary Responses to Adenoviral Delivery of CCL18 and Instillation of Bleomycin

To evaluate the combined effect of CCL18 overexpression and bleomycin injury on the lungs, each animal in this study received two intratracheal instillations; the first instillation of AdV-CCL18 or AdV-NULL or PBS was followed by the second instillation of bleomycin or PBS, as described in Materials and Methods. Mice instilled intratracheally with AdV-CCL18, AdV-NULL, or PBS showed no signs of morbidity such as body weight loss ($P > 0.05$, one-way analysis of variance), ruffled fur, dehydration, diarrhea, hunched posture, or decreased motor activity at any time postinfection. Mice instilled with bleomycin showed an expected total body weight loss of maximum $6.2 \pm 2.7\%$ following instillation of 0.01 U of bleomycin and maximum $10.1 \pm 3.2\%$ following instillation of 0.03 U of bleomycin. This statistically significant ($P < 0.05$ by one-way analysis of variance; data not shown) weight loss was observed on days 5 to 20 following the second intratracheal instillation of bleomycin but not PBS, independent of the nature of the agent used for the first instillation (AdV-CCL18, AdV-NULL, or PBS). There was minimal postoperative mortality that was not significantly different between animal groups (data not shown). Thus, bleomycin injury to the lung causes more severe morbidity manifested in weight loss than pulmonary CCL18 overexpression.

Histologically, instillation of AdV-CCL18 but not AdV-NULL or PBS caused perivascular and peribronchial lymphocytic infiltration (Figure 1, A and B); the T-lymphocytic nature of these cells was confirmed by immunohistochemical staining of lung sections for CD3, CD4, and CD8. Instillation of bleomycin caused the characteristic diffuse interstitial fibrosing alveolitis (Figure 1, C and D). The combined effect of CCL18 overexpression and bleo-

Figure 1. H&E staining of lung sections after intratracheal instillation of AdV-CCL18 (A and B), bleomycin (C and D), or their combination (E and F). Instillation of AdV-NULL caused a minimal infiltration of inflammatory cells on days 3 to 7 that was completely resolved by day 14 (data not shown). Instillation of AdV-CCL18 and subsequent CCL18 overexpression manifested in peribronchial and perivascular lymphocytic infiltration around small (A) and large (B) bronchi and vessels (arrows) and minimal interstitial lymphocytic infiltration (arrowheads). Instillation of bleomycin resulted in the characteristic diffuse interstitial alveolitis accompanied by distortion of alveolar architecture (C and D). The combined effect of CCL18 overexpression and bleomycin injury manifested in large areas of distorted alveolar architecture and more pronounced infiltration throughout the lung (E and F) and more severe interstitial lymphocytic infiltration remotely from bronchi and vessels (arrows). Immunohistochemical staining for CD4⁺ cells (G–I) confirmed the peribronchial and perivascular nature of the infiltration in the CCL18 overexpression model (G), more scattered presence of T cells in the lung parenchyma in the bleomycin injury model (H), and the combined lymphocytic accumulation pattern (adjacent to anatomical structures plus interstitial) on combined CCL18 overexpression and bleomycin injury to the lungs (I).

mycin injury exceeded the effect of each factor alone. It manifested in greater destruction of alveolar architecture, more pronounced perivascular and peribronchial infiltration, and massive lymphocytic accumulation not only in the peribronchial and perivascular areas but also in the interstitium (Figure 1, E and F). Immunohistochemical analyses for CD3, CD4, CD8, and T-cell receptor confirmed such infiltration patterns (an example for CD4⁺ cells is shown in Figure 1, G to I). Overexpression of CCL18 and bleomycin injury had an additive effect on T-cell content in BAL and total protein concentration in lung homogenates (Figure 2). Flow cytometric analyses of BAL cells revealed increases in T lymphocytes in CCL18-overexpressing mice as previously reported (see Figure 3 in ref. 11). Consistent with our previous report,¹¹

gating on CD3⁺CD4⁺ and CD3⁺CD8⁺ cells revealed the following CD4/CD8 ratios: 0.97 ± 0.17 in CCL18-overexpressing mice, 1.61 ± 0.22 in mice challenged with bleomycin, and 0.98 ± 0.09 in mice challenged with CCL18 overexpression and bleomycin injury ($n = 10$ in each group). In all cases, more than 90% of lung cells were immunohistochemically negative for proliferating cell nuclear antigen, suggesting that they are not dividing cells and that the observed dynamics of the infiltration is due to trafficking to the lungs (data not shown). Immunohistochemically and flow cytometrically, there was minimal (<1%) presence of CD19⁺ or B220⁺ cells. These observations together suggested that the bleomycin injury and overexpression of CCL18 together elicit a more severe proinflammatory effect on the lung than each of these

Figure 2. Absolute (A) and relative (B) BAL cell count and total protein concentration in lung homogenates. **A:** The majority of BAL cells were represented by macrophages and lymphocytes in all groups, with additive effects of CCL18 overexpression and bleomycin on total cell and lymphocyte counts (BLM, 0.03 U/mouse; shown). See Materials and Methods for description of the groups. **B:** Stacked column plot showing relative macrophage (top, open bars) and lymphocyte (bottom, shaded bars) content in bronchoalveolar lavage, mean percent $\pm$ SD of total BAL cells (the averaging procedure may lead to a combine cell counts slightly exceeding 100%). Other cell types were represented by neutrophils and epithelial cells and did not jointly exceed 3% in any of the groups; there was no difference in the neutrophil content between these groups ($P > 0.05$). As previously reported,¹¹ mice instilled with either PBS or AdV-NULL (Ctrl) showed no difference ($P > 0.05$). The second instillation was with either PBS or BLM as shown. The differences between Ctrl and CCL18 are significant in all cases ($P < 0.05$, Student's *t*-test, three to eight animals per group, repeated on three different occasions with consistent results). These data suggest that CCL18 overexpression and bleomycin injury have additive effect on lymphocytic accumulation in the lungs. **C:** Levels of total protein in lung homogenates in the combined CCL18 overexpression and bleomycin injury group exceeded those in any other group ($P < 0.05$), further suggesting that these two factors facilitate pulmonary inflammation in the additive fashion.

factors alone, particularly manifesting in additive accumulation of T lymphocytes in the lungs.

Some rare regulatory T cells (FoxP3⁺) were present in the infiltrates immunohistochemically; flow cytometric

analyses of the permeabilized BAL T cells revealed that $8.94 \pm 1.04\%$ of CD4⁺CD25⁺FoxP3⁺ were present in CCL18-overexpressing mice, whereas $8.48 \pm 1.22\%$ of such cells were present in mice overexpressing CCL18 in combination with bleomycin injury ($n = 5$ in each group, $P > 0.05$). Separate *in vitro* chemotaxis experiments using Transwell system were performed to separate CCL18-responding (lower chamber) from nonresponding (upper chamber) human T cells freshly purified from the peripheral blood mononuclear cell population. Flow cytometry analyses revealed that in both populations of T cells, for those that did or did not respond chemotactically to CCL18 the fractions of CD4⁺CD25⁺FoxP3⁺ cells were similar (6.95 ± 1.5 and $7.08 \pm 1.3\%$, respectively, $P > 0.05$). These *in vivo* data from animal models and *in vitro* data obtained with human purified T cells suggest that CCL18, alone or in combination with bleomycin, is not a selective attractor of regulatory T cells.

Figure 3. Total hydroxyproline per left lung (Hyp) as a surrogate measure of collagen content. **A:** Mean Hyp, $\mu\text{g} \pm \text{SD}$, three to eight animals per group. The second instillation was with either PBS or BLM as shown. Notice that in the absence of bleomycin, CCL18 overexpression stimulated collagen accumulation ($P < 0.05$). In contrast, CCL18 overexpression partially neutralized the effect of bleomycin on collagen accumulation ($P < 0.05$ for both doses of bleomycin). The expected additive effect of CCL18 overexpression and bleomycin injury on collagen accumulation is shown with the unfilled bars/dashed lines. **B:** Average Hyp level per lung in six independent experiments as described in A for BLM 0.03 U; standard deviations were similar to those shown in A (not shown in this panel to preserve clarity). Each point indicates average Hyp, presented as a percentage of Hyp value in corresponding PBS-treated controls, three to eight animals per group. The connecting lines represent six independent experiments performed on separate occasions.

Collagen Production and Accumulation in Pulmonary Fibrosis Models

Further experiments tested whether, similarly to the effect on T-lymphocytic infiltration, CCL18 overexpression and bleomycin in combination would enhance pulmonary fibrosis. In contrast to the additive effect of CCL18 overexpression and injury with bleomycin on the levels of T lymphocytes accumulating in the lungs, the CCL18 overexpression had a partially neutralizing effect on bleomycin-induced collagen accumulation (Figure 3). Consistent with previous observations,¹¹ overexpression of CCL18 by itself caused a moderate increase in total pulmonary hydroxyproline content compared with the increases caused by bleomycin alone (Figure 3A). However, the combined effect of CCL18 overexpression and bleomycin injury (black bars in the BLM groups in Figure 3A) was below the expected additive effect of these two factors (unfilled bars, dashed outlines in Figure 3A). Moreover, the combined effect of CCL18 overexpression and bleo-

mycin injury on collagen accumulation (black bars in the BLM groups in Figure 3A) was significantly lower than the effect of bleomycin injury alone (gray bars). This observation was made in six independent experiments, with consistent results (Figure 3B). An average decrease in total lung hydroxyproline (Hyp) was $33.4 \pm 3.9\%$, based on six independent experiments shown in Figure 3B. Thus, CCL18 overexpression causes T-cell-dependent¹¹ mild pulmonary fibrosis in an otherwise healthy lung,¹¹ yet it partially protects against the severe fibrotic injury caused by bleomycin (Figure 3). This effect was observed at different nonsaturating concentrations of bleomycin (Figure 3A) and at different times, on day 21 as described in Materials and Methods and shown in Figure 3, and on day 28 (not shown).

Possible Mechanisms of the Paradoxical Regulation of Collagen Levels in the Combined CCL18 Overexpression and Bleomycin Injury Model

We then considered possible mechanisms that might be involved in the unexpected regulation of collagen levels in the lungs in the combined CCL18 overexpression and bleomycin injury model. We focused on well-known regulators of connective tissue homeostasis, metalloproteinases MMP-2 and MMP-9,^{29–32} and major cytokines known to be involved in regulation of inflammation and fibrosis TGF- β 1, IL-13, TNF- α , IFN- γ , and MCP-1 (CCL2)³³ as possible regulators of the observed dynamics in collagen levels.

Levels of MMP-2 and MMP-9 are generally elevated in fibrotic lung diseases, these metalloproteinases are known to contribute both pro- and antifibrotically.^{29–32} The ELISA assays showed an additive effect of CCL18 overexpression on the levels of pro- and active MMP-2 and pro-MMP-9 in the lung homogenates (Figure 4, A and B). This additive effect was confirmed immunohistochemically for total (pro- and active) MMP-9 (Figure 4C). Zymographic analyses also revealed an increase in gelatinase activity in the combined CCL18 overexpression and bleomycin injury group (Figure 4D).

The levels of total TGF- β 1 and IL-13 measured by ELISA varied insignificantly between the groups ($P > 0.5$, Student's *t*-test, data not shown) similar to our previous report.¹¹ However, overexpression of CCL18 and bleomycin injury up-regulated the pulmonary levels of IFN- γ , TNF- α , and MCP-1 in an additive fashion (Figure 5A). Mediators IFN- γ and TNF- α have been shown to have both pro- and antifibrotic effects *in vivo*, with the profibrotic effects being secondary to inflammation, whereas *in vitro* these two factors are potent inhibitors of collagen production.³³ Thus, the increased levels of these cytokines provide a possible explanation for the decrease in collagen accumulation in the combined CCL18 overexpression and bleomycin injury model. Reciprocally, the levels of a known profibrotic regulator, active TGF- β 1, in the combined injury model were lower than in mice subjected to bleomycin

Figure 4. MMPs in the lungs of mice overexpressing CCL18 and/or treated with bleomycin. **A** and **B**: ELISA of lung homogenates for total (pro- and active) MMP-2 (**A**) and pro-MMP-9 (**B**). Data are shown as mean μg total protein $\pm$ SD, eight to 12 animals per group, repeated on two different occasions with similar results. Overexpression of CCL18 and injury with bleomycin act additively on accumulation of matrix metalloproteinases. In **A**, the differences between CCL18-expressing and -nonexpressing animals were significant ($P < 0.05$) in each bleomycin dose group. Similar tendencies were observed in **B**, although the differences between CCL18-expressing and -nonexpressing animals did not reach significance ($P > 0.05$) within each bleomycin dose group. **C**: Immunohistochemistry for MMP-9 (pro- and total), $\times 200$ magnification, showing the additive effect of CCL18 overexpression and bleomycin exposure on accumulation of MMP-9-producing cells in the lung (brown staining). No staining was detected with isotype control antibody (data not shown). **D**: Zymogram of lung homogenates. Sample loading was normalized to total protein (Bio-Rad assays). Molecular weight markers, kd, are indicated on the right. Expected locations of pro-MMP-9 (92 kd), active MMP-9 (82 kd), pro-MMP-2 (72 kd), and active MMP-2 (62 kd) are indicated with **arrows**. Repeated on three separate occasions in different groups of animals, with similar results.

injury alone (Figure 5D), thus mirroring the changes in hydroxyproline (see Figure 3). These observations suggest that multiple mediators, metalloproteinases, and cytokines are involved in the observed regulation of collagen levels in the combined CCL18 overexpression and bleomycin injury model.

Figure 5. ELISA of lung homogenates for IFN- γ (A), TNF- α (B), MCP-1 (C), and active TGF- β 1 (D), pg/ml, mean $\pm$ SD. Data were averaged from three to eight animals per group in A, B, and C and eight to 12 animals per group in D. The increases in IFN- γ (A), TNF- α (B), and MCP-1 (C) in the CCL18+BLM group were significant ($P < 0.05$) in comparison with any other group and seemed additive of the effects of CCL18 alone and BLM alone. In D, the level of active TGF- β 1 in the CCL18+BLM group was different ($P < 0.05$, Student's *t*-test) from any other group, but not additive of the effects of CCL18 alone and BLM alone. In these experiments, 0.03 U of BLM was used.

Effect of Lymphocyte Depletion and MMP Neutralization on Collagen Accumulation in the Lungs

We then sought to determine whether the presence of CCL18 is by itself sufficient to cause the observed changes in collagen accumulation or if there is a need for pulmonary T cells in mediating the observed effects. Treatment of mice with antilymphocyte serum before the administration of AdV-CCL18 did not affect CCL18 expression in the lung (real-time PCR and ELISA data not shown) but resulted in complete abrogation of the perivascular and peribronchial infiltration and collagen accumulation consistent with our previous report.¹¹ Consistent with previous reports of others,^{22,23,25} treatment with antilymphocyte serum did not have a significant effect on collagen accumulation in the bleomycin injury model ($P > 0.05$, Student's *t*-test, data not shown). In the combined CCL18 overexpression and bleomycin injury model treated with antilymphocyte serum, pulmonary levels of collagen did not differ from the bleomycin injury alone treated with antilymphocyte serum (205.8 ± 14.1 versus $199 \pm 12.3 \mu\text{g}/\text{lung}$, respectively, $P > 0.05$, Student's *t*-test, data not shown). Thus, the effect of bleomycin injury alone on collagen accumulation seems to be T-cell-independent, whereas depletion of T cells eliminates the effect of CCL18 overexpression on collagen accumulation in the normal or bleomycin-damaged lung.

The contribution of MMPs to tissue fibrosis seems complex.^{29–32} Therefore, we needed to determine whether the changes in the levels of MMPs (see Figure 4) contributed to regulation of collagen and cytokine levels. Administration of GM6001, a broad-spectrum pharmacological MMP inhibitor, significantly abrogated pulmonary levels

Figure 6. Changes in the total levels of hydroxyproline, fold increase versus control, in the lung of mice overexpressing CCL18 and challenged with a high dose of bleomycin on treatment with the MMP inhibitor GM6001 or neutralizing anti-MMP-9 antibody. Both treatments significantly abrogated accumulation of hydroxyproline and cytokine levels where indicated with asterisks ($P < 0.05$, Student's *t*-test, three to eight animals per group). Treatment with GM6001 in the group of mice instilled with 0.03 U of bleomycin alone did not attenuate the levels of hydroxyproline (207.3 ± 18.8 versus $222.7 \pm 15.6 \mu\text{g}/\text{lung}$, nontreated versus treated groups, respectively, $P > 0.05$).

of hydroxyproline, active TGF- β 1, TNF- α , and IFN- γ in the combined injury model (Figure 6), suggesting a central involvement of MMPs in these inflammatory and fibrotic processes. Administration of neutralizing anti-MMP-9 antibody had no effect on the levels of active TGF- β 1 or TNF- α but abrogated the levels of IFN- γ and further abrogated the levels of hydroxyproline, suggesting that MMP-2 is likely to be involved in regulation of TGF- β 1 and TNF- α levels. Thus, MMP-2 and MMP-9 contribute to the regulation of collagen and cytokine levels in the combined injury model.

Discussion

We have recently reported that mice infected with a replication-deficient adenovirus encoding CCL18 but not with a similar control virus develop selective T-lymphocytic infiltration of the lungs as well as moderate transient T-cell-dependent collagen accumulation.¹¹ Phenotypic characterization of the infiltrating cells in comparison with normally present pulmonary T cells revealed minimal, if any, activation, including lack of elevated expression of several profibrotic factors.¹¹ However, the lymphocytic infiltration coincided with the sites of accumulation of active TGF- β 1 and collagen,¹¹ suggesting that the infiltrating T cells directly contributed to the profibrotic effect of CCL18. In support of this notion, systemic depletion of T cells completely abrogated lymphocytic infiltration and collagen accumulation in CCL18-overexpressing mice.¹¹ In patients with lung fibrosis, increases in pulmonary levels of CCL18 occur in association with lung inflammation and fibrosis.^{1–7} Based on these observations, we hypothesized that a local inflammatory profibrotic milieu

might potentiate the T-cell-mediated profibrotic effect of CCL18.

Adenovirus-mediated gene delivery combined with bleomycin injury to the lung is an established approach that allows for testing this hypothesis.^{22,34,35} We first instilled mice with AdV-CCL18 as described¹¹ and then, at the peak of CCL18 production (day 7), performed a second intratracheal instillation with either PBS (as a control) or bleomycin in a lower (0.01 U/mouse) or higher (0.03 U/mouse) dose to induce lung inflammation and fibrosis. Of note, the selected doses of bleomycin alone were found to be insufficient to achieve a plateauing effect on lung inflammation and fibrosis in preliminary experiments (data not shown). The bleomycin challenge but not instillation of PBS caused a statistically significant transient loss of body weight on days 5 to 20 following the second intratracheal instillation (data not shown), independent of the nature of the agent used for the first instillation (AdV-CCL18, AdV-NULL, or PBS). Thus, bleomycin injury to the lung causes more severe morbidity manifested in weight loss than pulmonary CCL18 overexpression. The combined effect of CCL18 overexpression and bleomycin injury exceeded the effect of each factor alone on the severity of histological changes. It manifested in greater destruction of alveolar architecture, more pronounced perivascular and peribronchial infiltration, and massive lymphocytic accumulation not only in the peribronchial and perivascular areas but also in the interstitium (Figure 1). Overexpression of CCL18 and the bleomycin injury had an additive effect on T-cell content in the BAL samples and total protein concentration in lung homogenates (Figure 2). Thus, overexpression of CCL18 and the bleomycin injury together elicit a more severe proinflammatory effect on the lung than each of these factors alone.

In contrast to the additive effect on inflammation, CCL18 overexpression unexpectedly attenuated the severe bleomycin-induced collagen accumulation (Figure 3). This finding suggests that although CCL18-induced T-lymphocytic infiltration is by itself mildly profibrotic to a healthy lung,¹¹ the very same infiltration may be partially protective against severe fibrosis in a proinflammatory profibrotic setting in the lungs. Although a consistently reproducible phenomenon (Figure 3B), the amplitude of decline in pulmonary collagen level in the combined model compared with bleomycin alone is admittedly relatively modest, constituting $33.4 \pm 3.9\%$ of the normal collagen content. However, it is important to consider that fibrosis correlates with decline in lung function,³⁶ and that a therapy resulting in only 3% higher forced vital capacity than placebo is now being celebrated as a significant achievement in treating patients with scleroderma lung disease.³⁷ Our observation suggests that there might be potential for further enhancing the antifibrotic regulation in the lungs by therapeutically manipulating the local pulmonary milieu and/or the phenotypes of infiltrating T lymphocytes.

There are sporadic data on antifibrotic effects of T lymphocytes *in vitro*^{38,39} and *in vivo*.⁴⁰ The idiopathic pulmonary fibrosis patients with a higher percentage of BAL T lymphocytes may be more responsive to treatment.⁴¹ In combination with our findings, these data hint at the

possibility that T-cell infiltration in patients with pulmonary fibrosis is part of an antifibrotic feedback loop, raising a possibility that eliminating T cells from the inflamed lung may promote fibrosis and thus have a counterintuitive deleterious effect. Indeed, depletion of lymphocytes with antilymphocyte serum abrogated the perivascular and peribronchial infiltration and collagen accumulation (data not shown), consistent with our previous report.¹¹ Our previous data indicated that systemic antibody-mediated depletion of T lymphocytes completely abrogates the effects of CCL18 overexpression in the lungs.¹¹ Similarly, treatment with antilymphocyte serum in the combined CCL18 overexpression and bleomycin injury model did abrogate the partial protective effect of CCL18 overexpression in the combined model in immunocompetent mice; pulmonary levels of collagen in the T-lymphocyte-depleted animals that overexpressed CCL18 and were challenged with bleomycin did not differ from the immunocompetent mice challenged with bleomycin alone. Ultimately, further studies may prove that T lymphocytes may be therapeutically modulated to act antifibrotically instead of being targeted and eliminated from the lungs.

We began investigating the mechanism of the paradoxical regulation of the collagen accumulation in the combined CCL18 overexpression and bleomycin injury model. One possible mechanism might involve the regulatory T cell; however, no differences between the studied groups of animals were found in the content of CD4⁺CD25⁺FoxP3⁺ cells. In addition, *in vitro* chemotaxis assays revealed that CCL18 did not selectively attract such regulatory T cells. Together, these observations suggested that regulatory T cells explain the differences in collagen accumulation between animal groups in this study.

Metalloproteinases MMP-2 and MMP-9 are well-known regulators of connective tissue homeostasis that are involved in lung inflammation and fibrosis and act dually, proteolytically, and nonproteolytically in a complex concentration-dependent fashion.^{29–32} Depending on the specifics of the inflammatory milieu and local concentration of MMPs, their effects may be either pro- or antifibrotic. In this study, overexpression of CCL18 and injury with bleomycin acted additively on MMP-2 and MMP-9 levels and activity in the lungs. As part of addressing the mechanism of the observed phenomena, we also measured the levels of pro- and antifibrotic cytokines. The levels of proinflammatory cytokines TNF- α and IFN- γ were up-regulated by CCL18 overexpression and the bleomycin injury (Figure 5A) in an additive fashion. These two factors are known to have complex effect on tissue fibrosis, including their direct and indirect regulation of collagen production by fibroblasts.³³ One line of evidence, *in vitro* studies, suggests that they are potent direct inhibitors of collagen production in fibroblasts. In contrast, in some animal models each of these cytokines indirectly facilitated fibrosis through inflammation-dependent mechanisms.³³ The results on the elevation of these potentially antifibrotic cytokines that are presented here (Figures 5 and 6) are more consistent with their antifibrotic action, leading to a decline in collagen levels in the combined injury model (Figure 3). Reciprocally, the levels

of active TGF- β 1 declined in the combined injury model (Figure 5B), also consistent with the changes in collagen accumulation (Figure 3). Thus, the levels of the profibrotic factor active TGF- β 1 mirror those of collagen, whereas the levels of potentially antifibrotic factors TNF- α and IFN- γ change reciprocally, in agreement with the attenuation of the collagen content in the combined CCL18 overexpression and bleomycin injury model. Therefore, numerous factors are involved in the observed paradoxical regulation of collagen accumulation in the combined CCL18 overexpression and bleomycin injury model. Although TGF- β 1 is a central mediator of bleomycin-induced lung fibrosis, increases in the total levels of this powerful profibrotic cytokine are difficult to detect because of the overall high basal level of inactive TGF- β 1 (eg, see Figure 5C in Ref. 42). We, too, could not detect significant changes in total pulmonary TGF- β 1 in the studied models. There were no significant changes in the levels of a potent profibrotic cytokine IL-13 (data not shown).

Neutralization of MMPs with a broad-spectrum inhibitor GM6001 further attenuated the decline in collagen accumulation and the levels of the profibrotic and proinflammatory cytokines in animals with the combined CCL18 overexpression and bleomycin injury (Figure 6), confirming a significant role for MMPs and a potential for therapeutic modulation of these enzymes in lung fibrosis. A selective neutralization of MMP-9 with a specific neutralizing antibody attenuated the levels of collagen and IFN- γ but not TNF- α and active TGF- β 1, indicating that MMP-2 may be required for regulation of levels of the last two cytokines.

The novelty of these results is that the CCL18-mediated T-lymphocytic infiltration is profibrotic in the otherwise healthy lungs, but it is partially antifibrotic when superimposed on a second profibrotic injury (bleomycin). It also seems that the regulation of CCL18-mediated pulmonary inflammation and fibrosis is complex and occurs through mechanisms that involve T-lymphocytic infiltration, matrix metalloproteinases, and profibrotic and proinflammatory cytokines. The simplistic approach to developing new antifibrotic therapies in which pulmonary T lymphocytes are selectively targeted in hopes of diminishing the degree of fibrosis should be reconsidered, as these cells may play a partially protective role and, conceivably, could be phenotypically modulated to act even more antifibrotically.

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