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Profibrotic Activities for Matrix Metalloproteinase-8 during Bleomycin-Mediated Lung Injury

Vanessa J. Craig,* Pablo A. Quintero,*† Susanne E. Fyfe,* Avignat S. Patel,* Martin D. Knolle,*† Lester Kobzik,‡ and Caroline A. Owen*§†

Matrix metalloproteinase-8 (MMP-8) is a potent interstitial collagenase thought to be expressed mainly by polymorphonuclear neutrophils. To determine whether MMP-8 regulates lung inflammatory or fibrotic responses to bleomycin, we delivered bleomycin by the intratracheal route to wild-type (WT) versus Mmp-8−/− mice and quantified MMP-8 expression, and inflammation and fibrosis in the lung samples. Mmp-8−/− steady state mRNA and protein levels increase in whole lung and bronchoalveolar lavage samples when WT mice are treated with bleomycin. Activated murine lung fibroblasts express Mmp-8 in vitro. MMP-8 expression is increased in leukocytes in the lungs of patients with idiopathic pulmonary fibrosis compared with control lung samples. Compared with bleomycin-treated WT mice, bleomycin-treated Mmp-8−/− mice have greater lung inflammation, but reduced lung fibrosis. Whereas bleomycin-treated Mmp-8−/− and WT mice have similar lung levels of several pro- and antifibrotic mediators (TGF-β, IL-13, JE, and IFN-γ), Mmp-8−/− mice have higher lung levels of IFN-γ-inducible protein-10 (IP-10) and MIP-1α. Genetically deleting either Ifn-10 or Mip-1α in Mmp-8−/− mice abrogates their lung inflammatory response to bleomycin, but reconstitutes their lung fibroblastic response to bleomycin. Studies of bleomycin-treated Mmp-8−/− bone marrow chimeric mice show that both leukocytes and lung parenchymal cells are sources of profibrotic MMP-8 during bleomycin-mediated lung fibrosis. Thus, during bleomycin-mediated lung injury, MMP-8 dampens the lung acute inflammatory response, but promotes lung fibrosis by reducing lung levels of IP-10 and MIP-1α. These data indicate therapeutic strategies to reduce lung levels of MMP-8 may limit fibroproliferative responses to injury in the human lung. The Journal of Immunology, 2013, 190: 4283–4296.

Fibrosing lung diseases are some of the most devastating diseases of the lung. Among fibrosing lung diseases, idiopathic pulmonary fibrosis (IPF) is the most common and carries the poorest prognosis. Currently, we lack any effective treatments for this disease. The prevailing hypothesis for the pathogenesis of IPF is that an initial injury to the lung epithelium produces an aberrant and exuberant wound-healing process. A characteristic feature of IPF is the presence of fibroblastic foci, which are aggregates of proliferating fibroblasts and myofibroblasts, located beneath breaks in the epithelial basement membrane, which deposit interstitial collagens and other extracellular matrix (ECM) proteins in the lung.

ECM proteins (collagen, elastin, fibronectin, and laminin) accumulate in the lungs of patients with IPF. This process depends on the balance between lung levels of the following: 1) growth factors and profibrotic cytokines that activate fibroblasts and increase their production of ECM; 2) antifibrotic cytokines that inhibit ECM production by fibroblasts; and 3) proteinases, especially matrix metalloproteinases (MMPs), that degrade ECM proteins. Profibrotic growth factors include TGF-β, which stimulates fibroblasts to migrate, proliferate, transform into myofibroblasts, and synthesize interstitial collagens and other ECM proteins (1). Th2 cytokines (IL-4 and IL-13) and other mediators such as MIP-1α can also promote fibrotic responses to lung injury (2, 3). Antifibrotic mediators include IFN-γ and IFN-γ-inducible protein-10 (IP-10; CXCL10) (4). Mice genetically deficient in either IP-10 or a key IP-10 receptor (Cxcr3) by gene targeting have worse fibrosis, and Cxcr3−/− mice have higher mortality after bleomycin instillation when compared with wild-type (WT) mice (4, 5). Proteinases, especially MMPs, have important activities in regulating lung inflammatory and fibrotic responses to injury. MMPs cleave and thereby regulate the activities of proinflammatory mediators (6–10) and activate latent growth factors such as TGF-β (11, 12). In addition, MMPs degrade components of the ECM. The interstitial collagenase subfamily of MMPs [MMP-1, -8, -13, and -14 in humans; and MMP-8, -13, and -14 (13) in mice] are the key proteinases that degrade interstitial collagens (types I–III). As an interstitial collagenase, MMP-8 cleaves collagen at a single locus, and this cleavage step is rate limiting in collagen degradation (14, 15). Interstitial collagenases have been thought to limit fibrotic responses to injury based upon their potent collagen-degrading activities in vitro (15, 16), but these findings have not been confirmed in vivo.

MMP-8 (collagenase-2, neutrophil collagenase) is transcribed and translated in polymorphonuclear neutrophil (PMN) precursors in bone marrow (BM) in both humans and mice, and stored as latent pro–MMP-8 in the specific granules of mature PMNs (17–19). Upon PMN activation, pro–MMP-8 is released into the extracellular space, where it is activated by the cysteine switch mechanism.
MMP-8 has potent anti-inflammatory activities during LPS-mediated acute lung injury (ALI) and in allergic airway inflammation in mice (10, 23). During LPS-mediated ALI, MMP-8 reduces lung inflammatory responses to LPS by cleaving and inactivating MIP-1α (10). MMP-8 also reduces mortality in the hyperoxic model of lung injury in mice, which is mediated in part by MMP-8 cleaving and inactivating MIP-1α (10). In the OVA allogeneic murine model of allergic airway inflammation, MMP-8-deficient mice have increased granulocytic lung inflammation most likely due to reduced inflammatory cell apoptosis in the absence of MMP-8 (23). However, in a murine model of skin inflammation, MMP-8 has proinflammatory activity, and this is mediated by MMP-8 cleaving and activating the PMN chemokine, LPS-induced CXC chemokine (6, 24). MMP-8 also has crucial activities in wound healing because Mmp-8−/− mice have delayed neutrophil infiltration in full-thickness skin wounds, delayed resolution of inflammation, and delayed wound healing compared with WT mice due to altered TGF-β signaling (25). MMP-8 contributes to the generation of the neutrophil chemotaxtractant proline-glycine-proline (PGP), which promotes emphysema pathogenesis in mice (26, 27). Recently, an association was found between Mmp-8 gene variation and the extent of atherosclerosis in patients with coronary artery disease (28).

Although MMP-8 is a potent type I collagen-degrading protease, which might be expected to reduce lung fibrotic responses to injury, García-Prieto et al. (29) showed recently that MMP-8 reduces lung inflammation, but promotes lung fibrotic responses to bleomycin in mice by cleaving IL-10. Our previous work has shown that MMP-8 regulates the accumulation of PMNs and macrophages in the lung during LPS-mediated lung injury, at least in part, by cleaving and inactivating MIP-1α (10). In this study, we have built upon the prior studies of García-Prieto (29) by identifying which leukocyte subsets in the lung are regulated by MMP-8 during bleomycin-mediated ALI and the mechanisms involved. We also assessed whether MMP-8 regulates lung inflammatory and fibrotic responses to injury by reducing lung levels of MIP-1α and/or other mediators. Additionally, to identify the crucial cellular sources of MMP-8 in the lung mediating the activities of this protease in this model, we measured lung fibrotic response to bleomycin in MMP-8 BM chimeric mice. We found that bleomycin-treated Mmp-8−/− mice have higher lung macrophage and CD4+ T cell counts than bleomycin-treated WT mice. When compared with bleomycin-treated WT mice, Mmp-8−/− mice are protected from bleomycin-induced lung fibrosis and have reduced accumulation of myofibroblasts in the lung, and this is associated with higher lung levels of MIP-1α and IP-10 in bleomycin-treated Mmp-8−/− mice. Genetic deletion of either Ip-10 or Mip-1α in Mmp-8−/− mice reduces their lung inflammatory response to bleomycin, and restores their fibroproliferative responses to bleomycin. These data indicate that Ip-10 and Mip-1α are the key molecules in the lung regulated by MMP-8 during bleomycin-mediated lung injury. We have shown that both BM-derived leukocytes and lung parenchymal cells are crucial cellular sources of profibrotic MMP-8 during bleomycin-mediated lung injury. Our results indicate that strategies to inhibit MMP-8 activity or reduce MMP-8 levels in the lungs may limit lung fibrotic responses to injury. Thus, MMP-8 may be a novel therapeutic target for IPF and other fibrotic lung diseases.

**Materials and Methods**

**Materials**

Recombinant human MMP-8 and rabbit anti–MMP-8 IgG were purchased from Millipore (Billerica, MA). Murine MMP-8, human IP-10, and the ELISA kit for TGF-β were purchased from R&D Systems (Minneapolis, MN). The ELISA kit for measuring lung levels of MMP-8 in mice was purchased from MyBioSource (San Diego, CA). Murine rIL-4 and rIL-9 were the ELISA kits for measuring MIP-1α, IP-10, and IFN-γ were purchased from PeproTech (Rocky Hill, NJ). The ELISA kits for quantifying IL-13, IL-4, IL-9, and JE were purchased from eBioscience (San Diego, CA). The p-aminophenylmercuric acetate, 1,10 phenanthroline, Sigma proteinase inhibitor cocktail, PMSF, alkaline phosphatase–coupled monoclonal mouse anti-smooth muscle actin clone 1A4, Masson’s Trichrome stain kit, Bouin’s solution, Weigert’s iron hematoxylin solutions, and DTT were purchased from Sigma-Aldrich (St. Louis, MO). The Silver Xpress silver-staining kit was purchased from Invitrogen (Carlsbad, CA). Bleomycin was purchased from Henry Schein Animal Health (Melville, NY). Ketamine and xylazine were purchased from Webster Veterinary (Sterling, MA). The DNeasy DNA extraction kit was purchased from Qiagen (Valencia, CA). The Vector Red alkaline phosphatase substrate kit was purchased from Vector Laboratories (Burlingame, CA). Anti-CD4, IgG, CD8, and Gr-1 Abs were purchased from BD Biosciences (San Jose, CA). Goat anti-rabbit IgG–HRP conjugate was purchased from Bio-Rad (Hercules, CA).

**Animals**

All procedures performed on mice were approved by the Harvard Medical School Animal Care and Use Committee. All mice were housed in a barrier facility under specific pathogen-free conditions. Mmp-8−/− mice were generated in the mixed SVeV129×C57BL/6 skin (16) and backcrossed 10 generations into the pure C57BL/6 strain. The Mmp-8−/− mice have normal lifespan, fertility, and lung development, and no abnormality in the unchallenged state (6). We initially studied parental C57BL/6 wild-type littermate mice as our experimental controls for C57BL/6 Mmp-8−/− mice. Ip-10−/− mice in the pure C57BL/6 strain were purchased from The Jackson Laboratory (Bar Harbor, ME). Mmp-8−/−×Ip-10−/− mice were generated by crossing Mmp-8−/− and Ip-10−/− mice. Mip-1α−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mmp-8−/−×Mip-1α−/− mice were generated by crossing Mmp-8−/− and Mip-1α−/− mice.

**Genotyping**

The genotypes of all mice bred in house were confirmed by PCR-based genotyping protocols on DNA extracted from murine tails using Qiagen DNeasy extraction kits.

**Bleomycin-mediated ALI in mice**

Age- and gender-matched adult mice (10–16 wk old) were anesthetized with ketamine (200 mg/kg) and xylazine (10 mg/kg) and then given 60, 75, or 100 μl bleomycin in 30 μl endotoxin-free normal saline or 30 μl normal saline alone by the intratracheal (IT) route. Mice were euthanized by CO2 narcosis, followed by cervical dislocation at various intervals after IT bleomycin or saline. Preliminary experiments confirmed that the dose of bleomycin tested and the time points studied were optimal for each endpoint being studied. Twenty-one days after treating mice with 60 μl bleomycin, right lungs were inflated with PBS to 25 cm H2O and fixed in 10% (v/v) buffered formalin, and left lungs were snap frozen in liquid nitrogen and stored at −80°C for hydroxyproline assays or subsequently homogenized to quantify growth factors, cytokines, and chemokines using ELISAs. H&E and Masson’s Trichrome staining was performed on fixed and inflated lung sections. Three, 7, 10, and 14 d after treating mice with 75 μl bleomycin by the IT route, we performed bronchoalveolar lavage (BAL) and prepared lung digests and lung homogenates to measure lung inflammation. At intervals after treating mice with 100 μl bleomycin by the IT route, we prepared homogenates of lungs in PBS containing 0.5% Triton to measure IP-10 and MIP-1α using ELISAs, or extracted RNA from lung or BAL leukocyte samples to measure steady state Mmp-8 mRNA levels using real-time RT-PCR. To measure lung inflammation in bleomycin-treated mice, BAL was performed using eight 0.5-ml aliquots of sterile PBS. The BAL cell and supernatant fractions were separated by centrifugation (500 × g for 3 min). The BAL supernatant fractions were frozen to −80°C for quantitation of chemokines and cytokines using ELISAs. Erythrocytes were removed from the cell fraction by hypotonic lysis. Total and differential WBC counts were performed on the BAL leukocyte samples.

**Real-time RT-PCR to quantify Mmp-8 and IP-10 expression in lung samples**

Lungs were removed from WT mice 3, 5, and 7 d after delivering either 100 μl bleomycin or normal saline by the IT route. BAL was performed on WT mice 7–21 d after delivering 100 μl bleomycin or saline by the IT route to isolate lung leukocytes. Primary lung fibroblasts were isolated from the lungs of unchallenged WT mice, and cultured at 37°C until they were 80%...
confluent. Cells were then incubated at 37°C for up to 24 h with or without varying concentrations (1–10 ng/ml) of active human rTGF-β1. RNA was isolated from lung, BAL leukocytes, and murine lung fibroblasts using a TRIzol reagent method (30). Reverse transcription of the RNA samples was performed using a RETROscript kit from Applied Biosystems (Carlsbad, CA), according to kit instructions. Real-time RT-PCR analysis was performed using a Stratagene MPro instrument (Agilent Technologies, Santa Clara, CA) and a TaqMan Mmp-8 or Ip-10 gene expression assay (Invitrogen). We used the comparative cycle threshold method, cyclophilin, GAPDH, or 18S as endogenous reference genes, and FAM as the fluorophore.

Quantification of Mmp-8 protein levels in BAL fluid samples from mice

WT mice were treated with 100 μl bleomycin or saline, and 3, 7, 14, and 21 d later, BAL was performed using 1 ml PBS per mouse. BAL cells were removed by centrifuging the samples (500 x g for 5 min). Mmp-8 protein levels were measured in cell-free BAL fluid (BALS) samples using a commercially available ELISA kit.

Immunohistochemistry

Formalin-fixed lung sections from patients with IPF and from normal lung were deparaffinized. Ag retrieval was performed by incubating slides in boiling 0.01 M Tris containing 1 mM EDTA (pH 9.0) for 90 min. Slides were then blocked with 1% (v/v) BSA and 10% (v/v) goat serum in TBS (0.05 M Tris containing 0.15 M NaCl and 0.02 M CaCl₂) for 2 h at room temperature. Slides were then incubated with either rabbit anti–MMP-8 IgG or nonimmune rabbit IgG for 0.5 h at room temperature. Slides were then rinsed twice with TBS. Slides were then incubated in 3% hydrogen peroxide for 20 min, washed, incubated again with hydrogen peroxide, washed, and then incubated for 1 h at room temperature with goat anti-rabbit HRP conjugate (Bio-Rad) in serum block. Slides were then rinsed, incubated in avidin–biotin complex, and then incubated for 1 h at room temperature, rinsed again, and developed with 3,3'-diaminobenzidine. Slides were then counterstained with 1% (w/v) methyl green, dehydrated, and mounted.

Enzymatic lung digestion and cell staining

Lungs were removed from mice after flushing the right ventricle with 30 ml PBS, minced, and incubated with 0.1% (v/v) type IV collagenase and 0.05% (v/v) DNase for 1 h at 37°C. The digestion solution was then run through a 70-μm cell strainer, and red cells were removed using a hypotonic lysis step. Cells were then resuspended in 44% (v/v) Percoll, overlaid on 67% (v/v) Percoll, and centrifuged at 710 x g for 20 min. Cells were harvested from the interphase, washed, counted, and resuspended at 10 million cells/ml in PBS containing 2% (v/v) FBS with rat anti-mouse CD16/32 IgG2b, anti-CD4 IgG2b, and anti–Gr-1 IgG2b, or appropriate isotype-matched control Abs (PE-conjugated anti-rat CD4 IgG2b, anti–Gr-1 IgG2b, and FITC-conjugated anti–CD8 IgG2b or appropriate isotype-matched control Abs (PE-conjugated anti-rat IgG2b and FITC-positive conjugated anti-rat IgG2b)). The percentage of PE and FITC double-positive leukocytes was measured using flow cytometry after subtracting nonspecific staining detected on lungs fixed with different fixatives. Next, we isolated and studied the leukocytes from the lungs of bleomycin- versus saline-treated WT mice (Fig. 1B). WT and saline-treated mice were treated with either bleomycin or saline via the IT route. Lungs were removed at intervals and homogenized in PBS containing 150 mM NaCl, 20 mM CaCl₂, and 0.05% (v/v) Triton [pH 7.5] for 18 h at 37°C or buffer alone as a control. Reaction products were then separated on 16.5% (v/v) Tris Tricine gels at 60 V for 5 h and developed with a SilverXpress (Invitrogen) staining kit according to the kit manufacturer’s instructions.

BM chimeric mice experiments

WT and Mmp-8−/− recipient mice in the pure C57BL/6 background aged 10–16 wk were irradiated twice with 450 cGy 4 h apart. BM was isolated from WT or Mmp-8−/− donor mice, and 2 million BM cells were injected into the tail veins of each irradiated mouse in a volume of 20 μl PBS. Mice were housed for 8–10 wk to permit engraftment of BM, and Mmp-8 BM chimeric mice were then treated with either saline or 60 μl bleomycin by the IT route. Mmp-8 BM chimeric mice (WT BM donors into WT recipients, Mmp-8−/− BM donors into WT recipients, Mmp-8−/− BM donors into Mmp-8−/− recipients, and Mmp-8−/− BM donors into WT recipients) were euthanized 21 d after delivering either saline or 60 μl bleomycin via the IT route. The right lungs were inflated with PBS to 25 cm H₂O and fixed in 10% (v/v) buffered formalin. The left lungs were snap frozen in liquid nitrogen and stored at −80°C for hydroxyproline assay.

Statistics

Data are expressed as mean ± SEM or mean ± SD. The results for paired and unpaired data were compared by the Student t test for parametric data and the Mann-Whitney rank sum test for nonparametric data; p values <0.05 were considered significant.

Results

Mmp-8 expression in the lung is increased during bleomycin-mediated lung injury

To investigate whether Mmp-8 expression is regulated during bleomycin-mediated lung injury, we delivered bleomycin or saline by the IT route to WT mice, and 3, 5, and 7 d later, measured Mmp-8 steady state mRNA levels in whole-lung samples using real-time RT-PCR. Bleomycin-treated WT mice have a mean ~4-fold increase in steady state Mmp-8 mRNA levels in their lungs on day 7 compared with saline-treated WT mice (Fig. 1A). In addition, Mmp-8 protein levels in BALF samples significantly increased 7 d after delivering bleomycin, levels peak at 14 d, and Mmp-8 protein levels remain elevated 21 d after delivering bleomycin to WT mice (Fig. 1B).

Mmp-8 expression increases in murine lung fibroblasts activated with TGF-ß1 in vitro

Next, we investigated which cells in the lung produce Mmp-8 during bleomycin-mediated lung injury. We first attempted to immunostain lung sections from bleomycin-treated WT mice for Mmp-8. However, we were not able to detect specific staining for Mmp-8 in lung sections despite testing several commercial Abs on lungs fixed with different fixatives. Next, we isolated and studied leukocytes from the lungs of bleomycin- versus saline-treated WT mice as PMNs are known to be an important source of Mmp-8, and macrophages can be induced to express Mmp-8 transcripts when activated in vitro (32, 33). Although BAL leukocytes express Mmp-8 transcripts, steady state Mmp-8 mRNA levels did not significantly increase in BAL leukocytes isolated from the mice 7–21 d after bleomycin was delivered (Fig. 1C).

Human fibroblasts isolated from the synovium of patients with rheumatoid arthritis and gingival fibroblasts express Mmp-8 (34, 35), but it is not known whether lung fibroblasts express Mmp-8 in health or during pathologic processes. Accordingly, we mea-
sured Mmp-8 expression in primary murine lung fibroblasts that were either not stimulated or activated in vitro with an agonist that is relevant to the pathogenesis of IPF (active TGF-β1). Unstimulated murine primary lung fibroblasts express low levels of Mmp-8 mRNA transcripts, and levels do not increase over time when cells are incubated in the absence of agonists (as assessed by calculating ΔΔCt; data not shown). However, incubating fibroblasts with 10 ng/ml active rTGF-β1 induces ~4-fold increases in Mmp-8 steady state mRNA levels after 4 h (Fig. 1D). Time-course and dose-response experiments confirmed that our experimental conditions are optimal for inducing Mmp-8 expression in lung fibroblasts using TGF-β1 (Supplemental Fig. 1). Thus, Mmp-8 gene expression is likely to be upregulated in activated lung fibroblasts in bleomycin-treated mice.

**MMP-8 expression increases in leukocytes in the lungs of human IPF patients**

We immunostained lung sections from human IPF patients (n = 4) with an Ab to human MMP-8 or an isotype control-matched primary Ab. As a control, we immunostained sections of normal human lung tissue around surgically excised benign lung tumors (n = 2). We observed prominent staining for MMP-8 in leukocytes in the airways and the lung parenchyma in IPF lung samples. Representative immunostaining results are shown in Fig. 2. In contrast, there is minimal or no staining of leukocytes in the lungs of the IPF lung sections stained with the nonimmune control primary Ab, and no positive staining in normal lung sections stained with the anti–MMP-8 Ab (Fig. 2). Although bronchial epithelial cells are stained when we incubate IPF lung sections with the anti–MMP-8 Ab, similar staining is detected in bronchial epithelial cells stained with the nonimmune control Ab (as assessed by a senior pathologist, L.K.), indicating that this airway epithelial staining is not specific for MMP-8.

**MMP-8 promotes lung fibrosis during bleomycin-mediated lung injury**

To determine whether MMP-8 regulates lung fibrotic responses to lung injury, we compared lung fibroproliferative responses of WT versus Mmp-8−/− mice. As expected, there is robust staining for Mmp-8−/− mice harvested 21 d after the mice were treated with either bleomycin or saline by the IT route. Saline-treated WT and Mmp-8−/− mice have no evidence of pathology in H&E-stained lung sections, as expected (Fig. 3A). Bleomycin-treated WT mice have robust cellular infiltrates in their lungs after 21 d. However, the lungs of bleomycin-treated Mmp-8−/− mice are markedly less cellular than those of bleomycin-treated WT mice after 21 d. In addition, bleomycin-treated Mmp-8−/− mice have reduced lung collagen deposition after 21 d, as assessed by Masson’s Trichrome-stained lung sections (Fig. 3B) and quantitative hydroxyproline assays (Fig. 3D). Immunostaining of lung sections for α-smooth muscle actin (a marker of myofibroblasts) shows minimal staining in saline-treated WT and Mmp-8−/− mice, as expected. There is robust staining for α-smooth muscle actin in the lungs of bleomycin-treated WT mice, but this is markedly diminished in bleomycin-treated Mmp-8−/− mice (Fig. 3C). These results confirm those of an earlier study by García-Prieto et al. (29) that MMP-8 promotes lung fibrotic responses to bleomycin and increases lung myofibroblast numbers.

**MMP-8 reduces acute lung inflammation after bleomycin-mediated lung injury**

MMP-8 regulates the accumulation of PMNs and macrophages in the lung during LPS-mediated ALI (10). MMP-8 also increases total leukocyte counts 7 d after delivering IT bleomycin, but the leukocyte subsets regulated by MMP-8 in this model were not determined in our earlier study (10). Lung inflammation regulates lung fibrosis in the murine bleomycin model (36). Therefore, we quantified leukocyte subsets in the lungs of WT and Mmp-8−/− mice 3–14 d after delivering bleomycin or saline by the IT route.
Bleomycin induces a robust and complex lung inflammatory response in both WT and Mmp-8−/− mice, which peaks at 7 d and consists predominantly of macrophages (mean = 67 and 65% for bleomycin-treated WT mice and Mmp-8−/− mice, respectively) and lymphocytes (mean = 24 and 26% for bleomycin-treated WT mice and Mmp-8−/− mice, respectively). PMNs represent only ~9% of BAL leukocytes on average in both genotypes 7 d after IT instillation of bleomycin. However, lung inflammatory responses are significantly higher in bleomycin-treated Mmp-8−/− mice compared with bleomycin-treated WT mice on day 7 (Fig. 4A). The increased lung inflammatory response in bleomycin-treated Mmp-8−/− mice is predominantly due to higher BAL macrophage counts on day 7 in Mmp-8−/− mice (Fig. 4B). We also found a strong trend (p = 0.074) toward higher BAL lymphocyte counts on day 7 (Fig. 4C) in bleomycin-treated Mmp-8−/− mice versus WT mice, but BAL PMN counts are similar in bleomycin-treated WT and Mmp-8−/− mice at all time points examined (Fig. 4D). Lymphocytes (37–41) and PMNs (42–46) have crucial activities in regulating lung fibrotic responses to injury. However, PMN and lymphocyte counts are relatively low in BAL samples from the mice. Thus, we also quantified these leukocytes and lymphocyte subsets in lung digests from bleomycin-treated WT versus Mmp-8−/− mice.

MMP-8 increases CD4+ T cells in lungs after bleomycin-mediated lung injury

On day 7 after IT instillation of bleomycin, lungs from WT and Mmp-8−/− mice were enzymatically digested and stained with FITC for CD45, and with PE for CD4, CD8, or the PMN markers, Ly6C and Ly6G. Double-immunostained cells were then analyzed by flow cytometry. We found significantly higher numbers of CD4+ T cells in the lungs of bleomycin-treated Mmp-8−/− mice compared with bleomycin-treated WT mice (Fig. 5). However, bleomycin-treated WT and Mmp-8−/− mice do not differ significantly in the numbers of CD8+ T cells (data not shown) or PMNs in enzymatic lung digest samples (data not shown).

MMP-8 does not regulate lung levels of growth factors that promote lung fibrosis in bleomycin-treated mice

Next, we investigated the mechanism by which MMP-8 promotes lung fibrotic responses to injury. We considered the possibility that MMP-8 cleaves and thereby regulates the levels or biologic activities of growth factors or antifibrotic mediators in the lung. Growth factors such as TGF-β drive lung fibrotic responses to injury and are produced in latent forms that require activation to attain full activity (47–49). MMPs other than MMP-8 activate latent growth factors in vitro (11). Thus, we measured lung levels of latent and active growth factors and antifibrotic mediators in lungs removed from bleomycin-treated WT and Mmp-8−/− mice. Lung levels of several profibrotic mediators (total TGF-β and active TGF-β, IL-13, and JE) are similar in bleomycin-treated WT and Mmp-8−/− mice (Fig. 6A, 6B, Supplemental Table I). Lung levels of IL-4 and IL-9 are higher in bleomycin-treated Mmp-8−/− mice compared with bleomycin-treated WT mice (Supplemental Table I). There is a strong trend toward lower lung levels of IL-10 in Mmp-8−/− mice 48 h after instilling bleomycin, but WT and Mmp-8−/− mice do not differ in lung IL-10 levels 21 d after delivering bleomycin (Supplemental Table I). When we incubate purified active murine MMP-8 with murine rIL-4 or rIL-9, MMP-8 does not cleave these mediators as assessed by analysis of the reaction products separated on silver-stained Tris Tricine gels (Supplemental Fig. 2A, 2B). Thus, it is unlikely that MMP-8 reduces lung levels of IL-4 or IL-9 by proteolytically cleaving them.
increased lung levels of IL-4 and IL-9 in bleomycin-treated Mmp-8\(^{-/-}\) versus WT mice are quite modest in magnitude and could be secondary to the increased lymphocyte counts in bleomycin-treated Mmp-8\(^{-/-}\) mice because T cells produce these mediators (50, 51).

Mmp-8\(^{-/-}\) mice have higher lung levels of the antifibrotic mediator, IP-10, during bleomycin-mediated lung injury

To test whether MMP-8 promotes lung fibrosis by reducing lung levels of antifibrotic mediators, we measured lung levels of IP-10 and IFN-\(\gamma\) in lung homogenates from bleomycin-treated WT and Mmp-8\(^{-/-}\) mice. Bleomycin-treated WT and Mmp-8\(^{-/-}\) mice have similar lung levels of IFN-\(\gamma\) in lung homogenates (Supplemental Table I). Mmp-8\(^{-/-}\) mice have significantly higher IP-10 protein levels in lung homogenates than WT mice after they receive bleomycin (Fig. 6C), although the difference between the genotypes is modest in magnitude (<2-fold). When we measured IP-10 levels in BALF samples from the mice, we found a strong trend toward higher IP-10 protein levels in BALF from bleomycin-treated Mmp-8\(^{-/-}\) mice compared with BALF from WT mice (Supplemental Fig. 3). However, BALF IP-10 protein levels are low in both genotypes relative to levels detected in lung homogenates from bleomycin-treated WT and Mmp8\(^{-/-}\) mice. Measuring IP-10 protein levels in whole-lung or BALF samples potentially could dilute out substantial signals derived from specific groups of cells in the lungs. Thus, we also measured Ip-10 steady state mRNA levels in BAL leukocytes isolated from bleomycin-treated WT and Mmp-8\(^{-/-}\) mice at the time point corresponding to peak lung inflammation (day 7). BAL leukocytes from bleomycin-treated Mmp-8\(^{-/-}\) mice have substantially (~9.6-fold) higher Ip-10 steady state mRNA levels than BAL leukocytes from bleomycin-treated WT mice (Fig. 6D). Thus, MMP-8 potentially reduces the expression of Ip-10 in leukocytes recruited to the lung during bleomycin-mediated lung injury.

Genetic deletion of Ip-10 in Mmp-8\(^{-/-}\) mice reconstitutes their fibrotic lung responses to bleomycin-mediated lung injury

Lung levels of antifibrotic IP-10 are higher in bleomycin-treated Mmp-8\(^{-/-}\) mice. This could potentially explain the resistance of Mmp-8\(^{-/-}\) mice to bleomycin-induced lung fibrosis. We tested this possibility by genetically deleting Ip-10 in Mmp-8\(^{-/-}\) mice and comparing lung fibrotic responses to bleomycin in these Mmp-8\(^{-/-}\) \(\times\) Ip-10\(^{-/-}\) compound-mutant mice versus single-mutant mice. Genetic deletion of Ip-10 in Mmp-8\(^{-/-}\) mice reconstitutes the lung fibroproliferative response of Mmp-8\(^{-/-}\) mice to bleomycin. Lung collagen deposition in Mmp-8\(^{-/-}\) \(\times\) Ip-10\(^{-/-}\) mice is similar to that in bleomycin-treated WT mice as assessed by histology in H&E (Fig. 7A)- and Masson’s Trichrome (Fig. 7B)-stained lung sections, and quantitative hydroxyproline assays performed on lung hydrolysates (Fig. 7D). Genetic deletion of Ip-10 in Mmp-8\(^{-/-}\) mice also restores myofibroblast accumulation in their lungs (Fig. 7C). Thus, the profibrotic activities of Mmp-8 are due, at least in part, to MMP-8 decreasing IP-10 levels in vivo. When we tested the activity of active MMP-8 against IP-10 in vitro, we found that neither human MMP-8 nor murine MMP-8 cleaves IP-10 (a representative gel is shown in Supplemental Fig. 2C).
Genetic deletion of Ip-10 in Mmp-8−/− mice abrogates the increased BAL leukocyte counts observed in bleomycin-treated Mmp-8−/− mice

We also investigated whether IP-10 regulates inflammation during bleomycin-mediated lung injury in Mmp-8−/− mice because IP-10 is a chemokine for monocytes/macrophages, T cells, and NK cells (52–54), and the intensity of the lung inflammatory response to bleomycin can regulate subsequent lung fibrosis in this model (3, 37, 55, 56). Compared with bleomycin-treated WT mice, bleomycin-treated Ip-10−/− mice have similar total leukocyte, macrophage, PMN, and lymphocyte counts in BAL samples (Fig. 8). However, the increased total leukocyte count and the elevated...
macrophage, PMN, and lymphocyte counts in BAL samples in bleomycin-treated Mmp-8<sup>−/−</sup> mice are all attenuated by genetically deleting Ip-10 in Mmp-8<sup>−/−</sup> mice (Fig. 8).

Mmp-8<sup>−/−</sup> mice have increased lung levels of MIP-1α during bleomycin-mediated lung injury, but MMP-8 does not significantly regulate lung levels of other mediators that regulate lung inflammation.

We showed previously that MMP-8 limits lung inflammatory responses to LPS by cleaving and inactivating MIP-1α (10), which is a potent inducer of mononuclear cell migration (57) and PMN recruitment into the lung (58). Therefore, we tested whether MIP-1α protein levels in the lung are increased in the absence of MMP-8 during bleomycin-mediated lung injury. We found higher levels of MIP-1α protein in homogenates of lungs harvested from bleomycin-treated Mmp-8<sup>−/−</sup> mice compared with bleomycin-treated WT mice (Fig. 9A). However, levels of MIP-1α protein are below the lower limit of detection of the ELISA when measured in BALF samples from both WT and Mmp-8<sup>−/−</sup> mice. We also measured lung levels of other pro- and anti-inflammatory mediators (IL-13, IFN-γ, JE) and found no differences in lung levels of any of these mediators in bleomycin-treated WT versus Mmp-8<sup>−/−</sup> mice (Supplemental Table I). There is a strong trend toward lower lung levels of IL-10 in Mmp-8<sup>−/−</sup> mice compared with WT mice 48 h after IT bleomycin, but lung IL-10 levels are similar in WT and Mmp-8<sup>−/−</sup> mice 24 h and 21 d after IT bleomycin (Supplemental Table I).

Genetic deletion of Mip-1α in Mmp-8<sup>−/−</sup> mice abrogates the increased inflammation observed in bleomycin-treated Mmp-8<sup>−/−</sup> mice

To assess whether the higher lung levels of MIP-1α protein in bleomycin-treated Mmp-8<sup>−/−</sup> mice contribute to the increased lung inflammatory response of Mmp-8<sup>−/−</sup> mice to IT bleomycin, we compared lung inflammatory responses of WT, Mmp-8<sup>−/−</sup>, Mip-1α<sup>−/−</sup>, and Mmp-8<sup>−/−</sup> x Mip-1α<sup>−/−</sup> mice to IT bleomycin versus IT saline. Mip-1α<sup>−/−</sup> mice have a markedly attenuated lung inflammatory response to IT bleomycin, with reduced BAL macrophage, PMN, and lymphocyte counts (Fig. 9B–D). Both the increased total leukocyte and the increased absolute macrophage, PMN, and lymphocyte counts in BAL samples in bleomycin-treated Mmp-8<sup>−/−</sup> mice are significantly abrogated by genetic deletion of Mip-1α in Mmp-8<sup>−/−</sup> mice (Fig. 9B–D). These data indicate that MMP-8 restrains the lung inflammatory response to bleomycin, in part, by reducing lung levels of MIP-1α.

Genetic deletion of Mip-1α in Mmp-8<sup>−/−</sup> mice reconstitutes their fibrotic lung responses to bleomycin-mediated lung injury

Next, we investigated whether the protection of Mmp-8<sup>−/−</sup> mice from bleomycin-mediated lung fibrosis is linked to their higher lung levels of MIP-1α. Genetic deletion of Mip-1α in Mmp-8<sup>−/−</sup> mice reconstitutes their lung fibroproliferative responses to bleomycin as assessed by analysis of lung sections stained with H&E and Masson’s Trichrome stain or for α-smooth muscle actin, and hydroxyproline assays performed on lung hydrolysates (Fig. 10).

Both BM-derived cells and lung parenchymal cells are a source of profibrotic MMP-8 during bleomycin-mediated lung injury

To investigate whether BM-derived cells or lung parenchymal cells are a source of profibrotic MMP-8, we generated MMP-8 BM chimeric mice. When the BM in WT mice is replaced with Mmp-8<sup>−/−</sup> BM, there is a significant (but incomplete) reduction in the lung fibrotic response to bleomycin compared with WT recipients transplanted with WT BM (Fig. 11). When the BM in Mmp-8<sup>−/−</sup> mice is replaced with WT BM, the defective lung fibrotic response to bleomycin in Mmp-8<sup>−/−</sup> recipients is partially restored as assessed by analysis of lung sections from the mice stained with H&E (Fig. 11A) and Masson’s Trichrome stained (Fig. 11B), or for smooth...
muscle actin (Fig. 11C). There is also a strong trend toward a significant increase in lung collagen content in bleomycin-treated Mmp-8−/− recipients transplanted with WT BM compared with bleomycin-treated Mmp-8−/− recipients transplanted with Mmp-8−/− BM as assessed by quantitative hydroxyproline assays performed on lung hydrolysates (Fig. 11D). These results indicate that both BM-derived cells and lung parenchymal cells are sources of profibrotic MMP-8 during bleomycin-mediated lung injury.

Discussion

In this study, we have confirmed the recent finding of García-Prieto et al. (29) that MMP-8 promotes bleomycin-mediated lung fibrosis in mice. We show that MMP-8 expression is upregulated in lung samples during bleomycin-mediated lung injury and in murine lung fibroblasts activated with TGF-β ex vivo. Additionally, our novel data show that MMP-8 decreases the accumulation of macrophages and CD4+ lymphocytes in the lung during the acute phase of the bleomycin model by reducing lung levels of MIP-1α and IP-10. Our studies of MMP-8 BM chimeric mice show that both BM-derived cells and lung parenchymal cells are key sources of profibrotic MMP-8 during bleomycin-mediated lung fibrosis.

Prior studies have shown that levels of other members of the MMP family are increased in blood or lung samples from patients with IPF, and that these other MMPs contribute to lung fibrotic responses to injury in mice. For example, MMP-1, -7, and -8 protein levels are increased in plasma samples (59), and MMP-2 and -9 protein levels are elevated in BALF from patients with IPF versus control subjects without lung disease (60). MMP-3 mRNA and protein levels are increased in lung tissue from patients with IPF compared with controls (61). Furthermore, MMP-3, -7, and -13 promote lung fibrotic responses to injury in mice (61–63).

Mechanisms by which MMP-8 promotes lung fibrosis in mice

We investigated whether MMP-8 activates growth factors during bleomycin-mediated lung injury because other MMPs activate latent TGF-β in vitro (11), and MMP-8 promotes wound healing in murine skin by altering TGF-β signaling (25). However, we found no significant differences in lung levels of active or total TGF-β in bleomycin-treated WT and Mmp-8−/− mice. We now report that the profibrotic activity of MMP-8 during bleomycin-mediated ALI is linked to MMP-8 reducing lung levels of MIP-1α and IP-10 because bleomycin-treated Mmp-8−/− mice have higher lung levels of MIP-1α and IP-10 compared with bleomycin-treated WT mice, and genetic deletion of either Mmp-8−/− mice restores the lung fibroproliferative responses of these mice to bleomycin to levels similar to those observed in bleomycin-treated WT mice.

IP-10 (CXCL10) is expressed by IFN-γ-activated monocytes, fibroblasts, and endothelial cells (64) and has antifibrotic activities following bleomycin-induced lung injury by inhibiting chemotaxis of lung fibroblasts (4). Interestingly, IP-10 production is impaired in fibroblasts in the lungs of IPF patients due to defective histone deacetylation and hypermethylation, and this may contribute to lung fibrosis in patients with IPF (65). We detected modestly (but significantly) higher levels of IP-10 protein in homogenates of lungs and BALF samples from Mmp-8−/− mice compared with WT mice at early time points after delivering bleomycin. However, lung leukocytes from bleomycin-treated Mmp-8−/− mice have substantially (9.6-fold) higher Ip-10 steady state mRNA levels compared with lung leukocytes from bleomycin-treated WT mice at the time point corresponding to peak lung inflammation. Likely measuring IP-10 levels in whole-lung samples dilutes out more impressive signals generated by subsets of cells such as leukocytes in the lungs. The high lung levels of antifibrotic IP-10 in bleomycin-treated Mmp-8−/− mice most likely contribute to the protection of these mice from lung fibrosis by inhibiting myofibroblast accumulation in the lung. Consistent with this hypothesis, our study shows that myofibroblast numbers are lower in the lungs of bleomycin-treated
Mmp-8/−/− mice when compared with those in the lungs of WT mice, and the accumulation of myofibroblasts in the lungs of bleomycin-treated Mmp-8/−/− mice is restored by genetic deletion of Ip-10 in Mmp-8/−/− mice. Although MMP-8 cleaves other CXC motif chemokines such as LPS-induced CXC chemokine and MIP-1α (10, 24), we found no evidence that either human or murine MMP-8 cleaves human or murine IP-10 in vitro.

The increased lung levels of MIP-1α in bleomycin-treated Mmp-8/−/− mice also contribute to the protection of Mmp-8/−/− mice from bleomycin-mediated lung fibrosis compared with WT mice, because genetic deletion of Mip-1α in Mmp-8/−/− mice reconstitutes lung fibroproliferative responses of Mmp-8/−/− mice to bleomycin. A previous study reported that MIP-1α has profibrotic activities in the lung (3), but our study did not confirm this finding. It is possible that the exuberant acute inflammatory response in the lungs of bleomycin-treated Mmp-8/−/− mice (due to increases in lung levels of both MIP-1α and IP-10) limits subsequent progression to fibroproliferation in Mmp-8/−/− mice. Consistent with this hypothesis, genetic deletion of Ip-10 or Mip-1α in Mmp-8/−/− mice reduces lung inflammation in Mmp-8/−/− mice during the acute phase of bleomycin-mediated ALI and subsequently restores their lung fibroproliferative response to bleomycin in the subacute phase of the model. Lung inflammation is linked to lung fibrosis during bleomycin-mediated ALI because Ab-mediated PMN depletion in bleomycin-treated hamsters worsens the animals’ lung fibrotic responses to bleomycin (55). Thus, in bleomycin-treated Mmp-8/−/− mice, the increased lung levels of IP-10 and/or MIP-1α may inhibit lung fibrosis, in part, by increasing lung inflammatory responses to bleomycin, leading to increased clearance of provisional matrix by urokinase-type plasminogen activator, which is highly expressed by leukocytes (66, 67). Alternatively, the antifibrotic activities of IP-10 may exceed the profibrotic activity of MIP-1α in the lung, thereby leading to overall protection of Mmp-8/−/− mice from bleomycin-induced lung fibrosis. These possibilities will be investigated in our future studies.

**Mechanisms underlying the anti-inflammatory activities of MMP-8 during bleomycin-mediated lung injury**

Our novel studies of Mip-1α−/− × Mmp-8/−/− and Ip-10−/− × Mmp-8/−/− mice showed that the anti-inflammatory activities of MMP-8 in bleomycin-treated mice are due to MMP-8 reducing lung levels of MIP-1α and IP-10. MIP-1α is known to be a potent chemokine for macrophages (68) and lymphocytes (69), and IP-10 increases the accumulation of macrophages and lymphocytes in other models of lung inflammation (70). Our study provides evidence that the increased lung levels of MIP-1α and IP-10 in bleomycin-treated Mmp-8/−/− mice contribute to the increased lung macrophage and CD4+ T lymphocyte counts in these mice. Likely, MMP-8 reduces lung levels of MIP-1α in bleomycin-treated mice by directly cleaving and inactivating this chemokine in the lung because our prior studies showed that MMP-8 cleaves MIP-1α and reduces its chemotactic activity in vitro (10).
Macrophages express receptors for both MIP-1α (Ccr1 and Ccr5) and IP-10 (Cxcr3), and both mediators are produced by macrophages and induce recruitment and activation of monocytes and macrophages (70–72). Although our study shows that MMP-8 does not cleave IP-10, a possible explanation for MMP-8 reducing IP-10 levels in the bleomycin-treated lung is that MIP-1α is upstream of IP-10 in this model because MIP-1α activates macrophages, and activated macrophages are a significant source of IP-10 (73). In support of this concept, Mip-1a−/− mice have a severely blunted systemic inflammatory response in a trauma-hemorrhage model of shock, and this is associated with reduced serum levels of various proinflammatory mediators that are produced by macrophages (74). Although IP-10 levels were not measured in Mip-1a−/− mice with shock, it is likely that macrophage production of IP-10 is reduced in mice lacking MIP-1α (74). In bleomycin-treated Mnp-8−/− mice, it is likely that loss of MMP-8-mediated proteolytic inactivation of MIP-1α leads to enhanced MIP-1α–induced IP-10 generation in the lung associated with increased lung inflammation, but reduced progression to lung fibrosis in the subacute phase of the bleomycin model. This possibility will be the focus of our future studies.

Macrophage counts are increased in the lungs of bleomycin-treated Mnp-8−/− mice compared with bleomycin-treated WT mice. Although alveolar macrophages are activated in the lungs of IPF patients, and have the potential to promote lung fibrosis because they produce increased amounts of fibronectin and platelet-derived growth factor (75–77), more recent studies show that alveolar macrophages have multiple phenotypes with respect to fibrosis, with alternatively activated macrophages (M2 macrophages) promoting fibrosis and classically activated macrophages (M1 macrophages) promoting resolution of fibrosis (78, 79). M2 macrophages in the lungs of patients with obstructive lung disease have increased expression of MMP-2 and -7, but MMP-8 expression by these cells was not assessed (80). We also found increased accumulation of CD4+ T cells in bleomycin-treated Mnp-8−/− mice. Although CD4+ T cells have profibrotic activities during some tissue responses to injury (81), CD4+ T cells inhibit or promote renal fibrosis in mice depending on the context of CD4+ T cell activation (82). Future studies will investigate whether the increased lung CD4+ T cell or macrophage counts in bleomycin-treated Mnp-8−/− mice contribute to their protection from fibrosis and/or whether MMP-8 regulates the macrophage skewing from a M1 to a profibrotic M2 phenotype.
Cellular sources of profibrotic MMP-8 in the lung

We detected robust staining for MMP-8 protein in leukocytes in the lungs of patients with IPF. Although we did not detect significant increases in $Mmp-8$ steady state mRNA levels in leukocytes isolated from the lungs of bleomycin-treated WT mice, PMNs store MMP-8 protein within their specific granules, but do not synthesize MMP-8 de novo. It is likely that PMNs recruited to the bleomycin-injured murine lung contribute to the increases in MMP-8 protein levels that we detected in BALF samples. Macrophages represent most of the leukocytes present in BAL samples during bleomycin-mediated lung injury, and express $Mmp-8$ mRNA transcript when activated ex vivo (33). However, our results indicate that they do not significantly contribute to the increase in MMP-8 steady state mRNA levels in the lung during bleomycin-mediated lung injury because steady state mRNA levels do not increase significantly in BAL leukocytes isolated from bleomycin-treated WT mice. Although other studies have shown that MMP-8 is expressed by fibroblasts isolated from organs other than the lung (34, 35), we now report that lung fibroblasts can be induced to express MMP-8 when stimulated ex vivo with TGF-β. Although the concentration of TGF-β that we used to induce murine lung fibroblasts to express MMP-8 is relatively high (10 ng/ml), other studies have used this concentration to activate fibroblasts (83–85). Additionally, 10 ng/ml TGF-β is optimal for inducing human fibroblasts to express MMP-13 (86). This suggests that optimal TGF-β-mediated induction of collagenase gene expression (versus other genes regulated by TGF-β) in lung fibroblasts may require relatively high concentrations of TGF-β. We also show that both BM-derived cells and lung parenchymal cells are crucial sources of profibrotic MMP-8 during bleomycin-mediated lung injury based upon the lung fibroproliferative responses of MMP-8 BM chimeric mice to IT bleomycin. Potential BM-derived sources of MMP-8 include macrophages (33), PMNs (10), and fibrocytes (87). Our results also indicate that fibroblasts are potential sources of profibrotic MMP-8 among lung parenchymal cells.

Limitations of our study include the shortcomings of the bleomycin murine model as a model of IPF. For example, lung fibrosis induced by bleomycin eventually resolves in marked contrast to human IPF in which lung fibrosis is progressive. Bleomycin induces robust lung inflammation, whereas minimal inflammation is present in the lungs of most patients with IPF. Also, therapeutics that have efficacy in the bleomycin model in mice have not been effective in treating human IPF (88). Our future studies will evaluate $Mmp-8^{-/-}$ mice in newer models of lung fibrosis, including the repeat dosing bleomycin model that leads to persistent lung fibrosis (89), lung-specific overexpression of TGF-β models, and radiation-induced lung fibrosis models.

Other studies of MMP-8 in the context of fibrosis

In a murine model of oblitative bronchiolitis, MMP-8 has profibrotic activities associated with increased migration of PMNs into the airway lumen (90). However, in a model of liver fibrosis in rats, PMN-derived MMP-8 activity was increased during the repair phase of this model, and this was associated with resolution of fibrotic scarring (91). Recent studies from García-Prieto et al. (29) also report that MMP-8 has anti-inflammatory and profibrotic activities in the lungs of bleomycin-treated mice. Our study agrees with these findings, and adds to this literature by providing insights into the mechanisms underlying the anti-inflammatory activities of MMP-8 during bleomycin-mediated lung injury and the leukocyte subsets that are regulated in the lung by MMP-8. García-Prieto et al. (29) attributed the protection from lung fibrosis of bleomycin-treated $Mmp-8^{-/-}$ mice to higher lung levels of IL-10 in $Mmp-8^{-/-}$ mice than WT mice, which they detected 3 and 6 wk after instilling bleomycin. Although we found slightly lower lung levels of IL-10 in $Mmp-8^{-/-}$ mice 48 h after delivering bleomycin by the IT route, we found similar lung levels of IL-10 in WT versus $Mmp-8^{-/-}$ mice 3 wk after instilling bleomycin. It is noteworthy in this respect that there are conflicting prior reports in the literature on the effects of IL-10 in regulating lung fibrotic responses to injury as bleomycin-treated IL-10−/− and WT mice have similar lung fibrotic responses (92), but transgenic mice overexpressing IL-10 in the lung in an inducible manner develop lung fibrosis (93). Additional studies are needed to address the reasons for these conflicting reports underlying the activities of IL-10 in regulating lung fibrosis.

We now report that MMP-8 has anti-inflammatory and profibrotic activities during bleomycin-mediated lung injury in mice by decreasing lung levels of MMP-1α and IP-10. We have shown that both BM-derived cells and lung parenchymal cells are crucial sources of profibrotic MMP-8. Our data suggest that treatment strategies aimed at reducing lung levels of MMP-8 or inhibiting the profibrotic activity of MMP-8 in the lung may have therapeutic potential in IPF patients.

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Disclosures

The authors have no financial conflicts of interest.

References


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Table I. Lung levels of IL-13, 4, -9, and 10, and Ifn-γ, JE in WT versus Mmp-8−/− mice treated with IT saline or bleomycin.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Treatment</th>
<th>WT mice (pg/mL)</th>
<th>Mmp-8−/− mice (pg/mL)</th>
<th>WT versus Mmp-8−/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13</td>
<td>IT saline</td>
<td>114.35 (10.45)b</td>
<td>85.70 (6.48)</td>
<td>p = 0.333</td>
</tr>
<tr>
<td></td>
<td>IT bleomycin, 3 d</td>
<td>67.45 (4.13)</td>
<td>67.68 (3.81)</td>
<td>p = 0.976</td>
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<tr>
<td></td>
<td>IT bleomycin, 7 d</td>
<td>85.04 (11.48)</td>
<td>71.38 (3.45)</td>
<td>p = 0.202</td>
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<tr>
<td></td>
<td>IT bleomycin, 10 d</td>
<td>57.81 (3.69)</td>
<td>64.56 (6.23)</td>
<td>p = 0.344</td>
</tr>
<tr>
<td>IL-4</td>
<td>IT saline</td>
<td>71.63 (1.03)</td>
<td>72.76 (4.48)</td>
<td>p = 1.000</td>
</tr>
<tr>
<td></td>
<td>IT bleomycin, 24 h</td>
<td>62.02 (3.09)</td>
<td>90.08 (4.19)</td>
<td>p &lt; 0.001</td>
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<td>IT bleomycin, 48 h</td>
<td>55.13 (2.67)</td>
<td>85.13 (7.83)</td>
<td>p = 0.005</td>
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<tr>
<td>IL-9</td>
<td>IT saline</td>
<td>578.75 (14.55)</td>
<td>596.45 (32.25)</td>
<td>p = 0.667</td>
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<td>IT bleomycin, 24 h</td>
<td>548.30 (15.23)</td>
<td>685.25 (19.75)</td>
<td>p &lt; 0.001</td>
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<td></td>
<td>IT bleomycin, 48 h</td>
<td>501.18 (22.52)</td>
<td>678.63 (48.39)</td>
<td>p = 0.008</td>
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<tr>
<td>IL-10</td>
<td>IT saline</td>
<td>2220.00 (118.00)</td>
<td>1591.00 (12.00)</td>
<td>p = 0.333</td>
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<td></td>
<td>IT bleomycin, 24 h</td>
<td>1781.67 (73.51)</td>
<td>1967.67 (174.43)</td>
<td>p = 0.349</td>
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<tr>
<td></td>
<td>IT bleomycin, 48 h</td>
<td>1511.00 (48.92)</td>
<td>1354.00 (61.44)</td>
<td>p = 0.077</td>
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<tr>
<td></td>
<td>IT bleomycin, 21 d</td>
<td>1427.38 (72.41)</td>
<td>1286.50 (252.19)</td>
<td>P = 0.526</td>
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<tr>
<td>Ifn-γ</td>
<td>IT saline</td>
<td>140.35 (4.35)</td>
<td>64.03 (2.85)</td>
<td>p &lt; 0.001</td>
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<td></td>
<td>IT bleomycin, 3 d</td>
<td>60.05 (3.23)</td>
<td>58.07 (4.80)</td>
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<td>IT bleomycin, 7 d</td>
<td>62.59 (12.35)</td>
<td>65.97 (3.37)</td>
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<td>IT bleomycin, 10 d</td>
<td>49.79 (3.34)</td>
<td>53.99 (4.84)</td>
<td>p = 0.476</td>
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<tr>
<td>Je</td>
<td>IT saline</td>
<td>238.00 (32.20)</td>
<td>618.55 (71.35)</td>
<td>p = 0.333</td>
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<td></td>
<td>IT bleomycin, 24 h</td>
<td>945.77 (429.23)</td>
<td>1153.12 (461.96)</td>
<td>p = 0.240</td>
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<td></td>
<td>IT bleomycin, 48 h</td>
<td>773.75 (229.90)</td>
<td>697.13 (205.80)</td>
<td>p = 0.814</td>
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</tbody>
</table>
Table 1 Legend:

a WT and *Mmp-8*/*- mice treated with saline (n = 2-5 mice per group) or bleomycin (n = 5-18 per group) 24 h, 48 h, 3 d, 7 d, 10 d, and 21 d later lungs were removed. Lung homogenates were prepared and levels of Il-13, Il-4, Il-9, Il-10, Ifn-γ, and Je protein were measured using ELISAs.

b Data are mean (SEM).

c WT and *Mmp-8*/*- mice do not differ significantly in lung levels of IL-13, Ifn-γ, or JE at any time point studied after IT bleomycin.
Supplemental Figure 1: Activation of murine lung fibroblasts with different concentrations of TGF-β1. Fibroblasts were isolated from the lungs of WT mice and cultured until they were 80% confluent. Fibroblasts were then incubated for 2-24 h at 37°C with or without 10 ng/ml TGF-β1 (left panel) or varying concentrations of TGF-β1 for 24 h (right panel) and steady state mRNA levels were measured using qRT-RT-PCR. Mean ± SEM fold change in Mmp-8 gene expression was measured using GAPDH as the housekeeping gene and the ΔΔC_T method. In the left panel, n = 5-10 experiments/group for activated cells and n = 2-5 experiments/group for unstimulated cells. In the right panel, n = 3-6 cell preparations/group.
Figure 2: Mmp-8 does not cleave Il-4, Il-9, or Ip-10 in vitro.

Recombinant murine pro-Mmp-8 (0.96 μM) was incubated with 1 mM amino-phenyl mercuric acetate (to activate pro-Mmp-8) and with or without recombinant murine Il-4 (1.85 μM) (in A), recombinant murine Il-9 (1.75 μM) (in B). Recombinant human MMP-8 (0.17 μM) was incubated with 1 mM amino-phenyl mercuric acetate (to activate pro-MMP-8) and with or without recombinant human IP-10 (5 μM) (in C), as described in Methods. Reaction products were then separated on Tris Tricine gels at 60 V for 5 h and protein signals on the gel visualized using a silver staining kit. Note that when each mediator is incubated with the active proteinase, there is no generation of cleavage products of the mediators having lower molecular mass than that of the intact mediator.
Figure 3:  

**Mmp-8 decreases Ip-10 protein levels in BALF samples.** WT and $\text{Mmp-8}^{-/-}$ mice were treated 75 mU of bleomycin vs. saline by the IT route and 3-10 days later, BAL was performed and Ip-10 protein levels quantified in cell-free BALF samples using an ELISA. Data are mean ± SEM; n = 4-7 mice/group. Ip-10 levels were below the lower limit of detection of the assay in all the saline-treated control mice (data not shown).