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Opposing Regulatory Roles of Complement Factor 5 in the Development of Bleomycin-Induced Pulmonary Fibrosis

Erin Addis-Lieser,* Jörg Köhl,† and Mónica G. Chiaramonte2*

The mechanisms of idiopathic pulmonary fibrosis pathogenesis, a chronic and progressive interstitial lung disease, remain elusive. The complement system, a crucial arm of the innate immune response, plays a pivotal role in several pathological disorders; however, the contribution of individual complement components to lung fibrosis has not yet been examined. Complement factor 5 (C5) and its cleavage product C5a are critical mediators in inflammatory diseases. Thus, to evaluate the role of C5 in lung fibrosis, we compared congenic C5-sufficient and C5-deficient mice in a well-characterized murine model of bleomycin-induced pulmonary fibrosis. C5-deficient mice had an exaggerated inflammatory phenotype compared with C5-sufficient mice during acute bleomycin-induced lung injury. These findings suggest a protective and anti-inflammatory role for C5, which was linked to the regulation of matrix metalloproteinases involved in cell migration. In contrast, C5 had a detrimental effect during chronic stages of bleomycin-induced injury, indicating a profibrotic role for C5. This deleterious activity for C5 was associated with expression of the fibrogenic cytokine TGF-β1 and matrix metalloproteinase-3, an important mediator in fibroblast contraction. Altogether, our data reveal novel and opposing roles for C5 in both inflammation and tissue repair. Furthermore, these findings provide insight into the development of new therapeutic strategies for idiopathic pulmonary fibrosis patients.


Diopid pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown etiology, characterized by diffuse and progressive fibrosis, which leads to a dramatic loss of pulmonary function (1). This inflammatory and fibroproliferative disorder has an incidence of ~15 per 100,000/year in the U.S., and results in death in 50–60% of the cases (2). The mechanisms underlying IPF pathogenesis remain largely unknown, although etiologic factors such as viral insults, smoking, and genetic predisposition have been associated in the process (1). Although several studies have implicated a variety of cytokines, chemokines, and extracellular matrix components in the development of pulmonary fibrosis (3–5), the origin of tissue injury and the immunological components involved in the abnormal repair process observed in IPF are still unclear. Moreover, IPF patients respond poorly to the current therapeutic regimens (6); thus, a better understanding of this disorder is clearly warranted.

The experimental model of bleomycin-induced pulmonary fibrosis has been used extensively to elucidate the basis of pulmonary fibrosis (7). Administration of bleomycin to rodents induces an early inflammatory response, characterized by marked infiltration of neutrophils, eosinophils, and lymphocytes into the lung, followed by the development of interstitial fibrosis. This model has provided considerable insight into the immunological mechanisms involved in the pathogenesis of IPF, as well as other pulmonary fibrotic diseases.

The complement system is a crucial arm of the innate immune system. Once activated, it results in enzymatic cleavage of several proteins that form a complex network crucial for host defense. The activation of initial components of the cascade subsequently leads to the formation of complement factor 5 (C5) convertase, generating C5a anaphylatoxin and C5b from C5. Recent studies have demonstrated a critical role for C5 in the pathogenesis of autoimmune diseases, delayed-type hypersensitivity responses, experimental allergic asthma, and sepsis (8–11). Importantly, C5a has proinflammatory effects, as well as immunomodulatory properties (12). C5 exerts a potent chemotactic activity for neutrophils, macrophages, basophils, mast cells, and B and T cells (13–17). Furthermore, C5 contributes to diverse biological functions ranging from early hemopoiesis, skeletal and vascular development, liver regeneration, cell survival, and apoptosis (18, 19).

A potential contribution of the complement system to IPF pathogenesis has been suggested by studies in human IPF patients. Increased levels of immune complexes, which can directly activate the complement cascade, as well as complement fragments of the classical and alternative pathways, such as C3d, C4d, and BA, have been detected in the circulation and bronchoalveolar fluid of IPF patients (20–22), indicating extensive activation of complement pathways and a potential association with pulmonary pathogenesis in these patients. A potential role of the complement system in pulmonary fibrosis was previously suggested as complete depletion of downstream complement components attenuates bleomycin-induced fibrosis in mice (23). However, this experimental approach does not allow the identification of any individual component of the complement system potentially involved in the establishment of fibrosis.

Thus, to evaluate the specific contribution of C5 in the pathogenesis of IPF, we compared the development of bleomycin-induced...
pulmonary fibrosis in congenic C57B10.D2 C5-sufficient (C5S) and C5-deficient (C5D) mice (24). Our findings reveal unique and dual roles for C5 in the establishment of bleomycin-induced pulmonary fibrosis. Specifically, C5 appears to have a protective role during the initial inflammatory response by modulating matrix metalloproteinases (MMPs) involved in cell migration. The profibrotic role of C5, which is manifest during later stages of bleomycin-induced lung pathology, arises by regulation of the fibrogenic cytokine TGF-β1 and direct induction of MMP-3 production in fibroblasts.

Materials and Methods

Mice

Six- to 8-wk-old congenic C5S (B10.D2-H-2d H2-T1/8/0SnJ) and C5D (B10.D2-H-2d H2-T1/8/0SnJ) mice were obtained from The Jackson Laboratory. All mice were housed in Cincinnati Children’s Hospital Medical Center American Association for the Accreditation of Laboratory Animal Care-approved animal facility in accordance with National Institutes of Health standards.

Bleomycin treatment

Mice were treated with bleomycin (Blenoxane; Mead Johnson) by intratracheal instillation, as previously described (25). Doses of 0.1 U (5 U/kg) or 0.03 U (1.5 U/kg) of bleomycin in a volume of 50 μl were administered per mouse according to the experiment. Control animals were treated with the same volume of PBS. Mice were sacrificed at different time points for collection of different tissues. Right lung lobes were collected and inflated with 4% paraformaldehyde for histological analysis of tissue sections stained with H&E. For localization of collagen fibers within the lung tissues, Masson’s trichrome-specific staining was used. Bronchoalveolar lavage fluid (BALF) was collected by flushing the airways three times with 1 ml of PBS, and BALF supernatants were obtained after centrifugation for 15 min at 12,000 × g.

BB5.1 Ab treatment

The anti-mouse C5 mAb (BB5.1, IgG1) (26, 27) was administered intratracheally to C5S mice using a dose of 500 μg on days 3, 10, and 17 after bleomycin exposure (0.03 U/mouse). Mice were sacrificed on day 21 after bleomycin administration.

BALF gelatin zymography

The 10% SDS-polyacrylamide gels containing gelatin (Invitrogen Life Technologies) were used to identify proteins with gelatinolytic activity from BALF supernatants (containing 10 μg of protein). After electrophoresis in nonreducing conditions, gels were washed in renaturing buffer (Invitrogen Life Technologies) for 30 min at room temperature to remove SDS, followed by incubation in developing buffer overnight at 37°C optimized for proteolytic activity. Protease activity appeared as clear bands after staining with Coomassie blue R250 and destaining in solution of 10% acetic acid. Gels were photographed in GelDoc (Bio-Rad). Purified MMP-2 and -9 controls were obtained from Chemicon International.

RNA extraction/RT-PCR

Total RNA was extracted from lung left lobes collected in STAT-60 RNAzol, and cDNA was prepared by reverse transcription, as described (28). Total RNA was extracted from lung left lobes collected in STAT-60 RNAzol, and cDNA was prepared by reverse transcription, as described (28). mRNA levels were assessed by real-time RT-PCR, using iCycler (Bio-Rad). Values were normalized by mRNA levels of β-actin (housekeeping gene), and relative units were calculated as: (β-actin Ct – gene Ct)/β-actin Ct) × 100,000, where Ct is the cycle threshold. Primers for IL-1β, TNF-α, TGF-β1, collagen I, collagen III; MMP-2, -8, -9, -13, -3, and -12; and TIMP-1 were previously published (29–31).

Cytokine ELISAs

Lung homogenates were prepared from left lungs homogenized in PBS/ protease inhibitors by using Omni Homogenizer. IL-1β and TGF-β1 protein levels were measured in lung homogenates by capture ELISA, using R&D Systems reagents, following the manufacturer’s instructions. Levels of TGF-β1 were expressed as total TGF-β1 (active + latent) per milliliter of homogenate, after acidification of the samples.

Hydroxyproline (Hp) assay

To quantify collagen deposition, left lung lobes were hydrolyzed in 6 N of HCl for 18 h at 110°C, and Hp levels were quantified by colorimetric assay using Ehrlich’s reagent, as described (32).

Isolation of lung fibroblasts

Lung fibroblasts were isolated from left lung lobes, minced, subsequently treated with Liberase (Roche) for 1 h at 37°C, and filtered through 40-μm cell strainers. Adherent cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 25 mM HEPES, and 50 μM 2-MA (complete medium), in a humidified 5% CO2 air incubator at 37°C and passed every 3–4 days by dissociation of confluent cultures with 0.25% trypsin with 1 mM EDTA. For experiments, fibroblasts were used between passages 6 and 8 and seeded at 1.5 × 104 cells in 25-cm2 flasks. Confluent cultures were rendered quiescent by eliminating serum content from the medium for 24 h, and DNA was extracted by using StatPrep RNAzol for subsequent reverse transcription-PCR, as described above.

In vitro fibroblast studies

NIH3T3 murine fibroblasts were cultured in complete medium in a humidified 5% CO2 air incubator at 37°C. Cells from passages 4–8 were seeded at 5 × 104 in 24-well plates and cultured with medium without serum for 24 h before incubation with different doses of human c5a (Sigma-Al drich). Cell lysates were extracted at different time points by using lysis buffer (M-Per; Pierce Lab), according to manufacturer’s instructions. Twenty micrograms of protein from cell lysates were submitted to electrophoresis in 4–20% Tris-glycine gels (Invitrogen Life Technologies) under reducing conditions and transferred to nitrocellulose membranes. Blots were probed with goat biotinylated anti-mouse MMP-3 (R&D Systems) in 1/250 dilution in PBS/Tween 20 (0.05% BSA), followed by avidin-peroxidase (HRP) in a 1/10,000 dilution. Bands were visualized using ECL reagent (Immun-Star HRP; Bio-Rad). Purified human MMP-3 (R&D Systems) was used as a control. For control of protein load, sheep anti-γ-actin Ab (Chemicon International) was used in a 1/2000 dilution, followed by antio sheep IgG (Zymed Laboratories) as secondary Ab. Expression of C5αR (CD88) in the surface of NIH3T3 fibroblasts was determined by FACS analysis, using the specific mAb 2E8 (33) (kindly provided by J. Zwerin, Georg August University, Gottingen, Germany), by using a BD Biosciences FACSScan and CellQuest software.

Statistical analysis

For the survival experiments, statistical analysis was performed by GraphPad PRISM software using log-rank test to compare two groups. ANOVA test was used to compare multiple groups using STATView 4.5 software. Statistical significance was determined by p < 0.05.

Results

C5 protects from bleomycin-induced lethal lung injury

To investigate the involvement of C5 in the development of bleomycin-induced pulmonary fibrosis, we compared the responses to bleomycin exposure in congenic C57B10.D2 C5S and C5D mice. These mice have been used to analyze the role of C5 in several in vivo models (34, 35). First, we compared the response of both strains to intratracheal administration of a high dose of bleomycin (0.1 U/mouse), to evaluate their respective susceptibility to lethal lung injury. The absence of C5 markedly enhanced mortality, and 50% of C5D mice died as early as 5 days after treatment and none survived after 14 days. In contrast, only 40% of C5S mice died during this period (Fig. 1). When using a lower dose of bleomycin (0.03 U/mouse), all C5S and C5D mice survived as long as 28 days after treatment (data not shown). Thus, the intense detrimental response triggered by a high dose of bleomycin observed in C5D animals strongly suggests a protective role for C5 during bleomycin-induced acute lung injury.

Bleomycin-induced pulmonary inflammation is enhanced in C5D mice

To evaluate the mechanisms involved in the development of acute inflammation and chronic fibrosis induced by bleomycin, we used
a lower and nonlethal dose of bleomycin (0.03 U/mouse) and analyzed inflammatory and fibrotic parameters at days 3, 7, 14, and 21 posttreatment. As expected, histological evaluation of the lung tissue of C5S mice showed a strong cellular infiltration 7 days after bleomycin administration (Fig. 2A), compared with the PBS-treated controls. In contrast, the inflammatory response to bleomycin was more pronounced in congenic C5D mice, with extensive areas of cellular infiltration in the lung compared with C5S mice (Fig. 2A, middle and right panels). These results were confirmed by enumeration of total lung cells prepared after enzymatic tissue dissociation (C5S bleomycin, $6.4 \pm 0.6 \times 10^6$ vs C5D bleomycin, $9.2 \pm 0.9 \times 10^6$ cells per left lung; $p < 0.05$).

The pronounced inflammatory response generated early after bleomycin exposure in the absence of C5 was also associated with increased expression of proinflammatory cytokines in the lung. Indeed, mRNA levels of IL-1β and TNF-α in C5D mice were significantly higher than those observed in C5S mice (Fig. 2B) as measured by RT-PCR in the lung tissue on day 7 posttreatment. C5D mice also exhibited significantly higher levels of IL-1β protein in lung homogenates, when compared with C5S mice on day 7 posttreatment (Fig. 2B). Overall, the exacerbated inflammatory phenotype observed early after bleomycin exposure in C5D mice is strongly indicative of a protective role of C5 in acute lung injury.

Attenuation of bleomycin-induced pulmonary fibrosis in C5D mice

To determine the effect of C5 on bleomycin-induced chronic fibrosis, we assessed total lung collagen content by quantifying the levels of Hp, an amino acid specifically present in collagen protein. Bleomycin-treated C5S mice showed a progressive increase in total Hp levels in the lung, which was significantly higher during chronic stages of the response (days 14 and 21) (Fig. 3A). C5D mice also showed increased Hp levels compared with their controls, but surprisingly those levels were consistently reduced when compared with C5S bleomycin-treated group on days 14 and 21.
posttreatment (Fig. 3A). The difference in bleomycin-induced pulmonary fibrosis observed between the strains was also evident by histological evaluation of lung tissue, which showed decreased deposition of collagen fibers in C5D than C5S mice on day 14 after bleomycin administration (Fig. 3B, middle and right panels). These results were corroborated by analyzing mRNA expression of the fibrillar collagens types I and III, because bleomycin-treated C5D mice had significantly lower mRNA levels of both genes compared with bleomycin-treated C5S animals (Fig. 3C). Taken together, these results suggest that C5 has a detrimental profibrotic role during later phases of the response to bleomycin.

In vivo blockade of C5 attenuates bleomycin-induced fibrosis

To confirm the specific role of C5 in the development of bleomycin-induced fibrosis, we decided to inhibit C5 activity in vivo by using a specific Ab that prevents C5 cleavage into its fragments C5a and C5b (26, 27). Intratracheal treatment of C5S mice with the BB5.1 mAb prevented the development of bleomycin-induced fibrosis, as evidenced by significantly decreased lung levels of Hp (Fig. 4). Importantly, these Hp levels were indistinguishable from those observed in C5S mice treated with PBS (Fig. 4). These results, in conjunction with those obtained using C5D mice (Fig. 3), strongly support the important role of C5 in contributing to the lung fibrotic process induced by bleomycin.

C5 deficiency is associated with decreased expression of TGF-β1

The cytokine TGF-β1 has been previously shown to be critical in the establishment of bleomycin-induced pulmonary fibrosis (36), as well as other fibrotic processes (37). Therefore, we compared the expression of TGF-β1 induced in response to bleomycin administration in C5S and C5D mice. RT-PCR analysis of total lung tissue showed that the TGF-β1 gene was significantly induced in C5S bleomycin-treated mice on days 14 and 21 after treatment, compared with their controls (Fig. 5A). In contrast, no increase in TGF-β1 mRNA was observed in C5D mice on days 14 and 21 after bleomycin (Fig. 5A). More importantly, TGF-β1 mRNA levels in C5D mice were consistently lower than C5S mice on days 14 and 21 after bleomycin exposure (Fig. 5A). Moreover, levels of TGF-β1 protein in lung homogenates were significantly decreased in C5D mice compared with C5S mice (Fig. 5B). These results indicate that the diminished fibrotic process observed in the absence of C5 might be explained by the decreased expression of the fibrogenic mediator TGF-β1.

C5 modulates the expression of MMPs

To further investigate the effects of C5 on the fibrotic process, we evaluated the expression of matrix metalloproteinases (MMPs),
which have been extensively studied in several experimental models (38). The MMP family of genes primarily controls extracellular matrix degradation, which affects normal biological activities, as well as a variety of pathological disorders (38). MMPs are regulated at level of gene transcription, by latent proenzyme activation (39), and are inhibited by a family of proteins known as tissue inhibitors of metalloproteinases (TIMPs), which bind to MMPs’ active site and latent forms of MMPs (40).

Specifically, we compared the induction of MMP genes during the course of the response to bleomycin in both C5S and C5D mice. First, we analyzed mRNA expression of collagenases MMP-8 and MMP-13, which preferentially degrade fibrillar collagen types I, II, and III. C5S and C5D mice showed a similar pattern of expression for both genes in response to bleomycin, except on day 3 after exposure, when C5D mice exhibited significantly higher mRNA levels of both MMP-8 and -13 compared with C5S mice (Table I).

Next, we analyzed bleomycin induction of gelatinases MMP-2 and MMP-9, which degrade collagen IV, one of the main components of the basement membrane. MMP-2 mRNA levels were significantly lower in C5D mice compared with C5S mice only on day 14 after bleomycin, while no differences were observed at other time points (Table I). In the case of MMP-9, C5D mice had significantly higher levels of this gene in the lung compared with C5S animals on day 3 after bleomycin exposure, but both strains showed similar expression later on in the response (Table I). These results were confirmed at the protein level by zymography, which allowed us to monitor gelolytic activity in BALF. C5D mice have elevated MMP-9 enzymatic activity compared with only weak activity in C5S mice on day 3 after bleomycin exposure (Fig. 6). No significant MMP-9 activity was observed in BALF in either C5S or C5D mice at later time points (data not shown). Increased MMP-2 enzymatic activity (latent and active) was detected as early as day 3 (Fig. 6) and maintained during the course of the response to bleomycin in C5S and C5D mice (data not shown).

We analyzed the expression of MMP-12 (macrophage metalloelastase), which degrades elastin and basement membrane components and plays a crucial role in chronic obstructive pulmonary disease (41). Bleomycin-treated C5S and C5D mice showed similar induction of MMP-12 mRNA throughout the course of the response, except on day 3 after bleomycin exposure, when C5D mice had significantly higher levels than C5S mice (Table I).

MMP-3 (stromelysin-1) has been shown to play a relevant role in wound-healing process (42), as well as in the activation of other metalloproteinase genes (43). At early phases of the response to bleomycin (days 3–7), both strains had a similar induction of MMP-3 compared with the PBS-treated mice (Fig. 7). C5S mice showed a significant induction of MMP-3 at later time points after bleomycin exposure. However, the absence of C5 produced a significant decrease in MMP-3 mRNA levels during later stages of the response to bleomycin (Fig. 7), on days 14 and 21 posttreatment compared with C5S mice. These results suggest that C5 contributes to MMP-3 modulation during chronic stages of bleomycin-induced lung injury.

Finally, TIMP-1 mRNA levels were significantly higher in the lungs of C5D mice than C5S mice on day 3 after bleomycin exposure. However, no significant differences between the strains were observed at other time points (Table I).

**Reduced expression of interstitial collagens and MMP-3 in C5D lung fibroblasts**

Our results strongly suggest that C5 contributes to the establishment of chronic bleomycin-induced pulmonary fibrosis. To evaluate whether C5 deficiency intrinsically affects one of the main lung cell types involved in collagen deposition, we isolated lung fibroblasts from both C5S and C5D mice treated with bleomycin in vivo and analyzed mRNA expression of collagens I and III. Expression of these collagens in lung fibroblasts from C5D mice was significantly impaired compared with cells isolated from C5S animals (Fig. 8A). Furthermore, we found significantly reduced levels of MMP-3 mRNA (Fig. 8B) in C5D lung fibroblasts compared with cells from C5S mice. These results were confirmed by Western blot analysis, which showed increased production of MMP-3.
protein in C5S fibroblasts, but no induction of this protein in C5D cells (Fig. 8C). MMP-3, predominantly produced by fibroblasts (44), regulates fibroblast contraction and subsequently collagen production (45). Thus, our results strongly suggest that C5 regulates the tissue-remodeling process by directly affecting the activity and functionality of lung fibroblasts.

**C5a induces production of MMP-3 in murine fibroblasts**

To directly prove the involvement of C5 on MMP-3 induction, we used an in vitro assay using murine NIH3T3 fibroblasts. Specifically, we evaluated whether C5a has a direct effect on fibroblasts, thus affecting cell contraction and collagen production. Indeed, 3T3 fibroblasts express C5aR on their surface, as determined by flow cytometric analysis (Fig. 9A). Western blotting analysis of cell lysates showed that treatment with C5a for 24 h produced a dose-dependent increase in production of MMP-3 protein in fibroblasts (Fig. 9B). These results strongly indicate that C5 can modulate fibrosis and tissue remodeling through a direct induction of MMP-3 production in fibroblasts, thus affecting cell contraction and collagen production.

**Discussion**

To date, the involvement of specific components of the complement system in pulmonary fibrosis pathogenesis has not yet been addressed. Our studies using a murine model of bleomycin-induced pulmonary fibrosis reveal distinctive and novel roles for C5 in the modulation of lung tissue injury and remodeling process. Remarkably, C5 appears to have both an early protective anti-inflammatory role as well as a later detrimental profibrotic effect during bleomycin-induced lung injury.

The beneficial role of C5 was evident in lethal acute lung injury, as C5D mice showed significantly higher mortality after exposure to a high dose of bleomycin compared with C5S (Fig. 1). Because all C5D mice receiving this high dose of bleomycin succumbed before the onset of fibrosis, severe acute lung injury is the more likely cause of the rapid mortality observed in these animals. Moreover, a similar protective effect for C5 was observed after using a lower and nonlethal dose of bleomycin, which produced a response to injury that progressed from early acute inflammation to later chronic fibrosis. Indeed, early after exposure to this low dose of bleomycin, C5D mice exhibited an exacerbated inflammation, with marked cellular migration to the lung and high levels of proinflammatory cytokines IL-1β and TNF-α on day 7, compared with C5S mice (Fig. 2). Thus, we postulate that C5 limits the magnitude of the early inflammatory phase triggered by bleomycin-induced injury.

One potential mechanism for the effect of C5 on early bleomycin-induced inflammation can be found in the expression of a particular group of MMPs. MMPs and TIMPs are important mediators in both normal and pathological processes involving extracellular matrix remodeling (39). Both MMPs and TIMPs have been detected in IPF patients (47, 48) and the bleomycin-induced fibrosis model (49, 50); however, their specific role in pathogenesis is still unclear. Specifically, our studies showed that C5D mice exhibited increased levels of MMP-8, -13, -9, -12, and TIMP-1 compared with C5S mice on day 3 after bleomycin treatment (Table I), which

![FIGURE 6.](image-url)

**FIGURE 6.** Levels of MMP-2 and -9 in BALF from C5S and C5D mice after bleomycin treatment. BALF samples, collected on day 3 after bleomycin exposure, were run in 10% gelatin gels (10 μg of protein per lane). Zones of enzymatic activity appear as clear bands. C, MMP-2- and -9-purified controls. M, m.w. Lanes 1–4, C5S bleomycin treated; lanes 5–7, C5D bleomycin treated. Arrow, MMP-9 band (92 kDa).

![FIGURE 7.](image-url)

**FIGURE 7.** Expression of MMP-3 in C5S and C5D mice. Lungs were collected from both strains at different time points after PBS or bleomycin treatment, and mRNA levels of MMP-3 were measured by real-time RT-PCR. Values at day 0 are indicative of average values for PBS-treated controls. Values are expressed as mean ± SEM. †, Denotes significant difference between Bleo-treated groups.
prevailed the peak influx of inflammatory cells observed in the lungs of C5D on day 7 (Fig. 2A). Interestingly, our results are in agreement with previous findings demonstrating the role of MMPs in cellular migration, due to their collagenase activity (51), as well as their modulation of chemokines (52). For instance, high expression of MMP-8 correlates with increased migration of inflammatory cells in a murine model of hepatitis B (53). MMP-12 has been associated with the ability of macrophages to migrate through matrix (54). MMP-9 is a crucial mediator in cell migration, as shown in models of acute lung injury (55) and asthma (56). Furthermore, it has been shown that MMP-9-deficient mice exhibited a diminished degree of alveolar bronchiolization (57) after bleomycin administration, indicating that MMP-9 is important in facilitating migration of bronchiolar cells into regions of alveolar injury. Collectively, our findings suggest that the protective role for C5 during the early response to bleomycin may arise from the inhibition of MMP-8, -13, -9, -12, and TIMP-1, particularly crucial in cellular migration to the lung. Moreover, our results reveal for the first time the involvement of C5 in the regulation of MMPs.

The fact that C5 prevents an excessive early cellular infiltration in the lung is surprising considering the well-known chemotactant activities of the C5a fragment, which is generated from C5 during complement activation. However, an anti-inflammatory role for C5 has also been revealed in recent studies. For example, deletion of C5 or C5aR resulted in worsening of both pancreatitis and pancreatitis-associated lung injury (58). Furthermore, administration of C5a protects against glutamate-mediated neurodegenerative disorder (59). Although the mechanisms underlying the anti-inflammatory role of C5 remain unclear, some studies have suggested that C5a can produce a desensitization of different cell types to secondary cell chemoattractants, such as IL-8 and leukotriene B4 (60). Therefore, it is possible that C5 would protect against a robust early inflammatory response by decreasing the response to secondary chemokines in the injury site.

Importantly, our studies revealed an opposing role of C5 during the chronic phase of bleomycin-induced injury. The aggressive inflammatory response initially developed in C5D mice after bleomycin did not translate into chronic exaggerated fibrosis later on in the response. Quite the opposite, the absence of C5 resulted in a partial reduction in collagen deposition during the chronic stages of the response to bleomycin (Fig. 3). These results were confirmed by using a specific anti-C5 mAb (26). Indeed, in vivo blockade of C5 significantly attenuated the development of bleomycin-induced pulmonary fibrosis (Fig. 4). Altogether, our results support a critical role for C5 as a profibrotic mediator and suggest that the early inflammation and the late fibrosis induced by bleomycin are dissociated.

One mechanism by which C5 might participate in the chronic induction of bleomycin-induced pulmonary fibrosis is by modulation of TGF-β expression. Specifically, we found that the attenuated fibrosis observed in C5D mice was associated with decreased levels of TGF-β1 mRNA and protein in bleomycin (Fig. 5). The crucial role of TGF-β has been extensively studied in several fibrotic processes (37), and increased expression of TGF-β mRNA and protein has been detected in IPF patients (61) and in bleomycin-induced fibrosis (36). Our studies reveal a link between C5 and TGF-β regulation that could be potentially important for fibrotic processes in other tissues.

Several investigators have emphasized the role of MMPs and TIMPs in the structural tissue remodeling that characterizes the chronic fibrotic response observed in IPF patients (47, 48, 62) and the bleomycin-induced fibrosis model (49, 50). We found no major

**FIGURE 8.** Expression of interstitial collagens and MMP-3 in CSS and C5D mouse fibroblasts. Lung fibroblasts were isolated from CSS and C5D mice after bleomycin treatment, as described in Methods. mRNA expression of collagen I, III (A), and MMP-3 (B) was evaluated by real-time RT-PCR in triplicate. Values are expressed as mean ± SEM. * denotes significant difference. C, Western blotting of lung fibroblast lysates isolated as described in Materials and Methods from C5S-PBS mice (lane 1), CSS-bleomycin mice (lane 2), C5D-PBS mice (lane 3), and C5D-bleomycin mice (lane 4). Membranes were probed with antimouse MMP-3 Ab and anti-β-actin for control of protein load.

**FIGURE 9.** Induction of MMP-3 by C5a in murine fibroblasts. A, Detection of surface CD88 (C5aR) by flow cytometry in NIH3T3 fibroblasts. Thin line, isotype control; dark line, anti-C5aR mAb 20/70. MFI, mean fluorescence intensity. B, Western blotting of fibroblast cell lysates treated for 24 h with different doses of rC5a and probed with anti-mouse MMP-3 Ab.
differences between CSS and C5D mice regarding the expression of collagenases, gelatinases, or TIMPs during chronic stages of the response to bleomycin. However, C5D mice exhibited MMP-3 mRNA levels lower than CSS mice on days 14 and 21 after bleomycin exposure, suggesting that the role of C5 in chronic tissue repair is associated with its ability to regulate MMP-3 expression. Indeed, we demonstrated that the fragment C5a directly induces MMP-3 protein production in murine fibroblasts (Fig. 9B). MMP-3 belongs to the group of stromelysins, which degrade laminin, fibronecrtin, elastin, and nonhelical collagens IV and IX (63). MMP-3 has been shown to play a crucial role in osteo- and rheumatoid arthritis (64, 65) and coronary heart disease (66). These findings reveal for the first time a direct effect of the complement system in the intricate process of tissue repair and remodeling.

Another relevant role of MMP-3 in fibrosis has been discovered using models of wound healing, in which MMP-3 deficiency was associated with normal patients (67), thus contributing to the distortion of the lung architecture. Therefore, we hypothesize that the profibrotic role of C5 is directly linked to the ability of MMP-3 to promote fibroblast contraction. Indeed, our results show that lung fibroblasts from bleomycin-treated C5D mice have significantly diminished levels of collagens I/III (Fig. 8A) and MMP-3 compared with fibroblasts from CSS mice (Fig. 8, B and C). As the expression of these genes is closely related to fibroblasts’ contractile capacity, these results strongly validate the essential contribution of C5 to the tissue-remodeling process by direct modulation of MMP-3 expression. Finally, it is notable that MMP-3 is preferentially expressed in the lungs of susceptible C75BL/6 mice compared with resistant BALB/c mice after bleomycin administration (data not shown), strongly suggesting a crucial role for MMP-3 in lung fibrosis pathogenesis.

Our studies revealing opposing regulatory roles for C5 in the establishment of bleomycin-induced pulmonary fibrosis are in accordance with recent studies identifying divergent roles for several mediators in the fibrotic response. Specifically, IL-4 has been shown to have an early anti-inflammatory role for IL-4 as well as a profibrotic role during chronic phases of the response in the bleomycin model (68). Moreover, mice deficient in integrin αvβ6 developed exaggerated lung inflammation, but are protected from bleomycin-induced chronic pulmonary fibrosis (69). These findings challenge the notion that increased inflammation in response to injury directly correlates with increased fibrosis during chronic stages. The dissociation between inflammation and fibrosis is supported by the lack of efficacy of anti-inflammatory treatments in IPF patients, which emphasizes the relevance of fibrogenesis in both lung injury and the repair process in pulmonary fibrosis (1). In summary, our results highlight for the first time a significant contribution for C5 in the development of chronic fibrosis and disclose a new level of involvement of the innate immunity system in tissue repair. Our studies propose that C5’s modulation of bleomycin-induced inflammation and fibrosis occurs in a biphasic fashion. At the beginning of the response to an acute injury, C5 has an anti-inflammatory role by modulating cell migration most likely through regulation of MMPs. At later time points of the response, C5 promotes fibrosis by regulating TGF-β and MMP-3 expression. As an effective therapy for pulmonary fibrosis is critically needed, our studies suggest that the manipulation of the complement system, particularly as inhibitors of C5/C5a have become available (70, 71), is a plausible therapeutic alternative for IPF treatment, as well as other devastating fibrotic disorders.

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Disclosures

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References


