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The Role of the Th2 CC Chemokine Ligand CCL17 in Pulmonary Fibrosis

John A. Belperio, Maria Dy, Lynne Murray, Marie D. Burdick, Ying Y. Xue, Robert M. Strieter, and Michael P. Keane

Increasing evidence suggests that the development of pulmonary fibrosis is a Th2-mediated process. We hypothesized that the CC chemokines that are associated with a Th2 profile (CCL17 and CCL22) have an important role in the development of pulmonary fibrosis. We measured CCL17 and CCL22 during the pathogenesis of bleomycin-induced pulmonary fibrosis. We found that both CCL17 and CCL22 were significantly elevated through day 20 as compared with control mice. Peak expression of CCL22 preceded the peak levels of CCL17, as measured by real-time quantitative PCR. CCR4 is the receptor for CCL17 and CCL22 therefore, to further characterize the role of CCL17 and CCL22, we measured CCR4 mRNA in lung tissue of bleomycin-treated mice by real-time quantitative PCR. CCR4 was significantly elevated in bleomycin-treated mice as compared with control mice. Immunolocalization demonstrated that CCR4 was expressed predominantly on macrophages. Neutralization of CCL17, but not CCL22, led to a reduction in pulmonary fibrosis. Immunolocalization of bleomycin-treated lung tissue and human idiopathic pulmonary fibrosis tissue specimens showed that epithelial cells expressed CCL17. These findings demonstrate a central role for Th2 chemokines and the macrophage in the pathogenesis of pulmonary fibrosis and are further support for the role of a Th2 phenotype in the pathogenesis of pulmonary fibrosis. The Journal of Immunology, 2004, 173: 4692–4698.

There is evidence suggesting that the cytokine profile of the natural immune/inflammatory response determines the disease phenotype responsible for either resolution or progression to end-stage fibrosis. Supporting evidence is derived from studies demonstrating that IFNs, especially IFN-γ, have profound suppressive effects on the production of extracellular matrix proteins, such as collagen and fibronectin (10, 11). The administration of IFN-γ in vivo can cause a reduction of extracellular matrix in animal models of fibrosis (10–12). Furthermore we have shown that IL-12 attenuates bleomycin-induced pulmonary fibrosis in an IFN-γ-dependent manner (13). In contrast the Th2 cytokines, IL-4 and IL-13, induce the expression of fibroblast-derived type I and III procollagens in a similar magnitude as TGF-β (14). Similarly IL-13 has been shown to induce fibrosis by selectively stimulating production and activation of TGF-β (15). We have shown that IL-13 promotes bleomycin-induced fibrosis, in part, through the elaboration of CCL6 (9). Neutralization of IL-13 lead to a reduction in fibrosis and macrophage numbers (9). Similar use of an IL-13 immunotoxin chimeric molecule that antagonizes the effect of IL-13 lead to a reduction in bleomycin-induced pulmonary fibrosis (16).

Chemokines and their receptors are essential components of Th1- and Th2-mediated responses (17). CXCR3 and CCR5 are mainly expressed on Th1 cells whereas CCR3, CCR4, and CCR8 are more characteristic of Th2 cells (17, 18). These findings demonstrate that chemokines are important in the amplification and polarization of the immune response. There is increasing evidence that pulmonary fibrosis is predominantly a Th2-mediated process. Therefore we hypothesized that CCL17 and CCL22 acting via CCR4 would have an important role in the pathogenesis of pulmonary fibrosis. In this study we demonstrate that CCL17 has an important role in the development of pulmonary fibrosis via the recruitment of lymphocytes and macrophages. The predominant cell expressing CCR4 was the macrophage and neutralization of CCL17 led to reduced numbers of intrapulmonary macrophages as well as lymphocytes.
Materials and Methods

Reagents

Polyclonal neutralizing anti-CCL22 and anti-CCL17 Abs and polyclonal goat IgG were purchased from R&D Systems (Minneapolis, MN). Neutralizing capacity was tested by the ability to inhibit chemotaxis of CEM-NK6 cells to CCL17 or CCL22. Anti-CCR4 Ab was purchased from R&D Systems. Anti-Mac-3 Ab was purchased from BD Pharmingen (San Diego, CA). The “anti-riprotease” buffer for tissue homogenization consisted of 1× PBS with one Complete tablet (Boehringer Mannheim, Indianapolis, IN) per 50 ml.

Human tissue specimens

Human tissue specimens were obtained from consenting individuals in accordance with institutional review board approval. All had clinical and radiographic findings consistent with the diagnosis of IPF, and all had pathologic confirmation of the diagnosis of usual interstitial pneumonia made by open lung biopsy. None of the patients had been previously treated or were currently being treated with corticosteroids or other immunosuppressive agents.

Animal model of pulmonary fibrosis

Female CBA/J mice (6–8 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME). We used these strains of mice as they are a well-characterized inbred strain of mice that are susceptible to bleomycin-induced pulmonary fibrosis. Mice were maintained in specific pathogen-free conditions and provided with food and water ad libitum. To induce pulmonary fibrosis from bleomycin (Blenoxane, a gift from Bristol Myers, Evansville, IN) (1 U/kg) on day 0 as previously described (9, 19, 20). Control animals received only sterile saline as previously described (9, 19, 20). Briefly, mice were anesthetized with ketamine injected i.p. followed by intratracheal instillation of 1 U/kg bleomycin in 25 μl sterile isotonic saline. At various time points postinstillation, animals were euthanized, and both lungs were removed for homogenization as described below. In separate experiments bleomycin-treated mice were passively immunized by i.p. injection with 4 μg of anti-CCL17, anti-CCL22, or 4 μg of polyclonal goat IgG as a control, daily until day 8 before maximal fibrosis. All studies were approved by the University of California, Los Angeles, CA, institutional animal care and use committee.

Lung tissue preparation

Bleomycin- or saline-treated (control) lungs were homogenized and sonicated in antiprotease buffer using a method as previously described (9, 19, 20). Control animals received only sterile saline as previously described (9, 19, 20). Tween 20 ha t4

Chemokine ELISA

CCL17 and CCL22 were quantitated using an ELISA as previously described (21). Briefly, flat-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 μg of the appropriate polyclonal Ab (1 ng/μl in 0.1 M NaCl, 0.26 M HIBu, and 0.08 N NaOH, pH 9.6) for 24 h at 4°C and then washed with PBS, and 0.05% Tween 20 (wash buffer). Non-specific binding sites were blocked with 2% BSA. Plates were rinsed and samples were added (50 μl/well), followed by incubation for 1 h at 37°C. Plates were then washed and 50 μl/well of the appropriate biotinylated polyclonal Ab (3 ng/μl in wash buffer, and 2% FCS) added for 45 min at 37°C. Plates were washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 min at 37°C. Chromogen substrate (DAKO, Carpinteria, CA) was then added, and the plates were incubated at room temperature to the desired extinction. Plates were read at 490 nm in an automated microplate reader (Bio-Tek Instruments, Winooski, VT). Standards were log(1/2) dilutions of recombinant chemokine (50 μl/well). Sensitivity of the assay was >50 pg/ml for each chemokine. Our ELISA was highly specific with no cross-reactivity to a panel of cytokines, including IL-1Ra, IL-1, IL-2, IL-4, IL-6, TNF-α, IFN-γ, CXC chemokine ligand 9, and other members of the CXC and CC chemokine families.

Real-time quantitative PCR for cytokine gene expression

Total lung RNA was extracted from mouse lungs using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) according to manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed into cDNA and amplified using TaqMan Reverse Transcription Reagents (PE Applied Biosystems, Foster City, CA). To exclude amplification of genomic DNA, samples were also run in the absence of reverse transcription. Real-time quantitative PCR was performed using the ABI Prism 7700 Sequence Detector and SDS analysis software (PE Applied Biosystems). For CCL17, CCL22, and CCR4 and 18S Realtime (Pe Applied Biosystems) were used for amplification. Reactions were assembled in 96-well reaction plates using TaqMan Universal PCR Master Mix (2X); the appropriate Pre-Developed Assay Reagent; and template (cDNA). PCR was performed under the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles at 95°C for 15 s, and 60°C for 1 min. Negative controls (no template cDNA) were performed on each PCR plate. Quantitative analysis of gene expression was done using the comparative C(T) (ΔC(T)) methods, in which C(T) is the threshold cycle number (the minimum number of cycles needed before the product can be detected) (23). The arithmetic formula for the ΔC(T) method is described as the difference in threshold cycles for a target (i.e., CCL17 or CCL22) and an endogenous reference (i.e., our housekeeping gene 18S). The amount of target normalized to an endogenous reference (i.e., CCL22, CCL17 or CCR4 in bleomycin-treated mice) and relative to a calibration normalized to an endogenous reference (i.e., CCL17, CCL22, or CCR4 in saline-treated mice) is given by 2 ΔΔC(T) (23). The calculation of 2 ΔΔC(T) then gives a relative value when comparing the target to the calibrator, which we designate in this context as fold increase in bleomycin-treated mice as compared with saline-treated controls (9).

Immunohistochemistry

Paraffin-embedded tissue from control and bleomycin-treated lung was processed for immunohistochemical localization of CCR4, CCL17, or macrophages (Mac-3), using a method as previously described (21). Similarly, paraffin-embedded tissue from human IPF or control lung was processed for immunohistochemical localization of CCL17 as previously described (24, 25). Briefly, tissue sections were dewaxed with xylene, and rehydrated through graded concentrations of ethanol. Tissue nonspecific binding sites were blocked using normal goat serum (BioGenex Laboratories, San Ramon, CA). Tissue sections were overlaid with 1/500 dilution of either control (rabbit) or polyclonal (rabbit) anti-CCL17, CCL22, or CCR4 Abs (R&D Systems) or control (rat) or polyclonal (rat) anti-Mac-3 (BD Pharmingen) Abs. The tissue sections were washed in Tris-buffered saline and then incubated for 60 min with secondary goat anti-rabbit biotinylated Abs (BioGenex Laboratories) or goat anti-rat biotinylated Abs. The tissue sections were then washed in Tris-buffered saline and incubated with alkaline phosphatase conjugated to streptavidin (BioGenex Laboratories). Tissue sections were then incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) followed by the peroxidase substrate, DAB reagent (Vector Laboratories, Burlingame, CA). After optimal color development, tissue sections were immersed in sterile water, counterstained with Lerner’s hematoxylin, and cover slipped using an aqueous mounting solution.

Collagen assay

Total lung collagen was measured using the Sircol Collagen assay (Bio-color, Belfast, Northern Ireland) according to manufacturers instructions and as previously described (26–28). Briefly, lungs were harvested and homogenized as described earlier, Sircol dye (1000 μl) was added to 100 μl of collagen standard or test samples (lung homogenate) and placed on a mechanical rocker for 30 min. Samples were then centrifuged at 5000×g for 5 min to pack the collagen dye complex at the bottom of the tube. Unbound dye solution was then removed and 1 ml of 0.5 M NaOH solution was added to the remaining pellet of collagen-bound dye. Following solubilization, samples were transferred to a 96-well plate and read at 540 nm on a Beckman DU-640 spectrophotometer (Fullerton, CA).

Assessment of infiltrating pulmonary leukocytes

Bronchoalveolar lavage (BAL) was performed to obtain cells for FACS analysis of leukocyte subsets. The trachea was exposed and intubated with a 1.7-mm outer diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1 ml aliquots. Three milliliters of PBS were instilled per mouse. Cell counts and viability were determined using trypan blue exclusion on a hemocytometer. Single cell suspensions were stained with anti-CD45 Tri-Color (Caltag Laboratories, South San Francisco, CA) to allow gating on CD45-positive cells for analysis of leukocytes. For analysis of lung leukocyte cell populations the following Abs directly conjugated to FITC or PE (BD Pharmingen) were used, CD3, CD4, CD8, NK1.1, Gr-1, and Moma-1. Cells were analyzed on a FACSscan flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences).

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The Journal of Immunology
Fibroblast Proliferation

Murine lung fibroblasts were cultured as previously described (19, 24). Briefly, murine pulmonary fibroblasts were grown to 80% confluence and passaged. At the time of the fourth passage, pulmonary fibroblast purity was >99% as determined by the absence of nonspecific esterase, Factor VIII-related Ag, or cytokeratin immunostaining. The cells were >90% positive for vimentin, laminin, and fibronectin and were >90% negative for α-smooth muscle actin and desmin. This technique allowed the establishment of murine pulmonary fibroblast cell lines. On the day of use, pulmonary fibroblasts were plated out in 96-well plates at a concentration of 5000 cells/well and allowed to adhere overnight. Fibroblasts were then washed free of serum and cultured for 24 h under serum-free conditions. At 24 h 5% FCS was again added along with varying concentrations of CCL22, CCL17, or platelet-derived growth factor (PDGF), and fibroblasts were cultured for a further 72 h. [3H]Thymidine incorporation was assessed using a Beckman LS 1801 scintillation counter (Beckman Instruments, Schaumburg, IL) and expressed as cpm.

Statistical analysis

Data were analyzed on a Dell computer using the Statview 5.0 statistical package (Abacus Concepts, Berkeley, CA). Comparisons were made using the unpaired t test. Data were considered statistically significant for values p ≤ 0.05.

Results

Lung tissue from mice treated with bleomycin has increased levels of CCL22 and CCL17

We measured CCL17 and CCL22 in the bleomycin-induced pulmonary fibrosis model. Total RNA was isolated from lungs of bleomycin-treated mice or saline-treated controls. Using quantitative PCR we found that CCL17 was significantly elevated at days 2, 8, 12, and 20 as compared with saline-treated controls (Fig. 1A). Furthermore lung tissue from bleomycin-treated mice demonstrated greater levels of CCL17 protein as assessed by ELISA (Fig. 1B). Similarly using quantitative PCR we found that CCL22 was significantly elevated at days 2, 4, 8, 12, and 20 (Fig. 1C). Lung tissue from bleomycin-treated mice also demonstrated greater levels of CCL22 protein as assessed by ELISA (Fig. 1D). Interestingly, peak levels of CCL17 as compared with CCL22 were 40-fold greater as measured by ELISA (Fig. 1, A and D).

CCR4 is elevated in bleomycin-induced pulmonary fibrosis

Because CCL17 and CCL22 share a common receptor, CCR4, we next assessed lung tissue from bleomycin-treated or saline-treated control mice at each time point for the presence of CCR4 by real-time PCR. Lung tissue from bleomycin-treated animals demonstrated greater levels of CCR4, as compared with saline-treated controls at days 2, 4, 8, 12, and 20 (Fig. 2). These findings suggested that CCR4 might be playing a role in the pathogenesis and the development of pulmonary fibrosis. To further characterize the role of CCR4, we performed immunolocalization for CCR4 in bleomycin-treated lung tissue. As shown in Fig. 3, CCR4 is predominantly expressed on macrophages. This localization to macrophages was confirmed with staining for Mac-3 (Fig. 3).

**FIGURE 1.** CCL17 and CCL22 are significantly elevated in bleomycin-treated as compared with saline-treated controls. A, Time course of CCL17 expression as measured by real-time quantitative PCR from lung tissue of mice exposed to either intratracheal bleomycin or saline control at day 0. Data are presented as fold increase in bleomycin-treated lung tissue as compared with saline-treated controls (n = 6 in each group). B, Time course shown of CCL17 production in lung tissue of mice exposed to either intratracheal bleomycin or saline control at day 0. CCL17 was measured by specific ELISA from lung tissue and normalized to nanograms per lung (n = 6 in each group). C, Time course of CCL22 mRNA as measured by real-time quantitative PCR from lung tissue of mice exposed to either intratracheal bleomycin or saline control at day 0. CCL22 was measured by specific ELISA from lung tissue and normalized to ng per lung (n = 6 in each group).
Neutralization of CCL17 but not CCL22 attenuates bleomycin-induced pulmonary fibrosis

To further investigate the role that CCL17 and CCL22 were playing in the pathogenesis of bleomycin-induced pulmonary fibrosis we next assessed whether neutralization of CCL17 or CCL22 by passive immunization would attenuate pulmonary fibrosis. Passive immunization with specific neutralizing Abs to CCL17 every 24 h for 8 days led to a significant reduction in pulmonary fibrosis, as compared with control Ab-treated mice as assessed by collagen levels (Fig. 4). Interestingly, using a similar strategy of neutralization of CCL22 had no effect on pulmonary fibrosis (Fig. 4). Neither anti-CCL17 nor anti-CCL22 have any effect on collagen content in the lungs of unchallenged mice (data not shown).

Passive immunization with neutralizing CCL17 Abs is associated with reduced BAL leukocyte and macrophage numbers

Having shown that neutralization of CCL17 attenuates bleomycin-induced fibrosis, we subsequently set out to determine the potential mechanisms for this effect. Single cell suspensions were prepared from BAL fluid of bleomycin-treated mice who had received either anti-CCL17 Abs or control Abs as previously described. Cellular populations were studied by FACS analysis at day 8. Passive immunization with neutralizing anti-CCL17 Abs led to a significant reduction in CD3, CD4, and CD8 lymphocytes, NK cells, macrophages, and neutrophils, as compared with controls, at day 8. (Fig. 5)

CCL17 does not stimulate fibroblast proliferation

Having shown that anti-CCL17 reduces fibrosis we were next interested to see whether CCL17 had any direct effect on pulmonary fibroblasts. Pulmonary fibroblasts were isolated from CBA/J mouse lungs and stimulated with various concentrations of CCL17, whereas PDGF was used as a positive control. Proliferation at 72 h was measured using incorporation of tritiated thymidine. As shown in Fig. 6, CCL17 had no effect on fibroblast proliferation. Furthermore we found no evidence of CCR4 mRNA expression from isolated fibroblasts as assessed by real-time PCR. These findings are further evidence for a role for CCL17 in the pathogenesis of bleomycin-induced pulmonary fibrosis other than through an effect on fibroblast proliferation, specifically leukocyte and macrophage recruitment.

FIGURE 3. Representative photomicrograph of the immunolocalization of CCR4 and macrophages in lung tissue at day 16 postintratracheal bleomycin administration (magnification, ×312). A, Bleomycin-treated lung tissue immunostained for CCR4 demonstrating localization to macrophages is shown. B, Same lung specimen immunostained with control Abs demonstrating the lack of nonspecific staining. C) Same lung specimen immunostained with the macrophage specific marker Mac-3. D, Same lung specimen immunostained with control Abs demonstrating the lack of nonspecific staining.

FIGURE 4. Lung collagen levels at day 16 from mice administered intratracheal bleomycin (day 0) and treated with either anti-CCL17, anti-CCL22 Abs, or control Abs. Abs were administered by daily i.p. injection until day 8. Saline-treated mice received no Ab (n = 6 lungs in each group).

FIGURE 2. Time course of CCR4 expression as measured by real-time quantitative PCR from lung tissue of mice exposed to either intratracheal bleomycin or saline control at day 0. Data are presented as fold increase in bleomycin-treated lung tissue as compared with saline-treated controls (n = 6 in each group).
Immunolocalization of CCL17 is predominantly associated with epithelial cells in bleomycin-induced fibrosis and human IPF lung tissue

Having demonstrated an important role for CCL17 in bleomycin-induced pulmonary fibrosis we were next interested to know what the predominant cellular source of CCL17 was in the lungs of mice treated with bleomycin. Furthermore we were interested to know whether CCL17 was expressed in human IPF lung tissue and if so, what was the predominant cellular source. Using immunohistochemistry, we found that the predominant cells in bleomycin-treated lungs and IPF lung tissue that expressed CCL17 were epithelial cells (Figs. 7 and 8).

Discussion

The natural history and sequence of events that dictate the pathogenesis of pulmonary fibrosis is not well characterized. Numerous factors that regulate immune and inflammatory responses have been implicated in the pathogenesis of pulmonary fibrosis. Regardless of the initial inciting agent, the hallmark of pulmonary fibrosis is chronic inflammation and deposition of extracellular matrix. The phenotype of this chronic inflammation appears to be highly associated with a Th2 phenotype of cytokine expression (29).

Th1 and Th2 cytokines are expressed by a variety of cells and the function of these cytokines are different, suggesting that an imbalance in the expression of Th1 and Th2 cytokines may be important in dictating different immunopathological responses (30, 31). Although there is a pattern for the existence of both Th1 (characterized by the expression of IFN-γ) and Th2 cytokines in IPF lung tissue, the presence of Th2 cytokines predominated over the expression of IFN-γ (29). This pattern of cytokine expression may be related to the potential role for the humoral response in the pathogenesis of IPF, or may be related to the inability of IFN-γ to tilt the balance away from Th2-dependent profibrotic environment. In further support of an imbalance of the presence of Th2 cytokines, as compared with IFN-γ, is the finding that IFN-γ levels are inversely related to the levels of type III procollagen in the BAL fluid of IPF patients (32).

Chemokines and their receptors are essential components of Th1- and Th2-mediated responses (17). CXCR3 and CCR5 are mainly expressed on Th1 cells whereas CCR3, CCR4, and CCR8 are more characteristic of Th2 cells (17, 18). We found elevated levels of the Th2 chemokines, CCL17 and CCL22, and their shared receptor, CCR4, in the bleomycin model of pulmonary fibrosis. This supports a role for these CC chemokines in the development of fibrosis. Previous researchers have shown that CCL17 is elevated in BAL fluid of patients with eosinophilic pneumonia that correlated with the presence of IL-4, IL-5, and IL-13 in BAL fluid (33). Similarly Katoh et al. (34) have shown that CCR4+CD4+ T cells in BAL fluid from patients with eosinophilic pneumonia correlated with BAL fluid levels of CCL17, CCL22, and IL-5. This suggests an important role for CCL17 in the recruitment of Th2 cells to the lung. Further support for this notion is seen in the report of increased expression of CCR4 on lymphocytes in the airway mucosa of atopic asthmatics (35). This was associated with the expression of CCL17 and CCL22 in airway epithelial cells upon allergen challenge (35). Furthermore, CCL22 has been shown to be inducible in bronchial epithelial cells (36). Similarly we found that CCL17 localized to the epithelium both in the bleomycin model and IPF tissue specimens. Interestingly it has been shown that CCR4-expressing lymphocytes were lacking in the airways of patients with sarcoidosis (35). Sarcoidosis is considered to be a predominantly Th1-mediated disease, and T cells infiltrating the airways of patients with sarcoidosis were found to

FIGURE 6. Murine pulmonary fibroblast proliferation at 72 h in response to media alone (control), platelet-derived growth factor (PDGF, 200 ng/ml), CCL17 (1, 10, 50 and 100 ng/ml). [3H]Thymidine was added to the cultures 12 h before fibroblast harvest. [3H]Thymidine incorporation was expressed as cpm. Data represent mean ± SEM, n = minimum of six in each group.

FIGURE 7. Photomicrograph showing immunolocalization of CCL17 from bleomycin-treated lung tissue specimens. A, Bleomycin-treated lung section (magnification, ×312) stained with anti-CCL17 Abs demonstrating immunolocalization to epithelial cells. B, Bleomycin-treated lung section (magnification, ×312) stained with control Abs demonstrating the lack of nonspecific staining.

FIGURE 5. FACS analysis of BAL leukocyte populations at day 8 in saline-treated mice or bleomycin-treated mice that received either anti-CCL17 or control Abs. Bleomycin or saline was administered at day 0 by intratracheal injection. Abs were administered by daily i.p. injection until day 8. Saline-treated mice received no Ab (n = 6 lungs in each group).
express IFN-γ and CXCR3 (35). In a model of chronic fungal asthma, the absence of CCR4 was associated with increased clearance of Aspergillus and the attenuation of many of the Th2 cytokine-associated features of this particular disease with decreased airway responsiveness at day 30 (37). There was however no difference in the airway remodeling that is seen with chronic Aspergillus infection (37).

Neutralization of CCL17 reduced pulmonary fibrosis. Interestingly neutralization of CCL22 had no effect on fibrosis. This might seem surprising given that both CCL22 and CCL17 bind to CCR4. However peak gene expression and protein levels of CCL17 were 10- and 40-fold higher, respectively, than CCL22. This would suggest that in this model of pulmonary fibrosis CCL17 is playing a more important role than CCL22. Another possibility is that CCL17 is acting independently of CCR4. Currently there is no evidence of an alternative receptor for CCL17. Neutralization of CCL17 is acting independently of CCR4. Currently there is no evidence of an alternative receptor for CCL17. Neutralization of CCL17 was associated with a decrease in inflammatory cells including macrophages. Furthermore macrophages were the predominant cells expressing CCR4. This supports the notion that the macrophage is a key inflammatory cell in the development of fibrosis. In human IPF lung tissue it has been shown that insulin like growth factor-1 immunolocalizes to alveolar macrophages and interstitial macrophages (38). The degree of clinical impairment and collagen deposition in the interstitium correlated with the degree of staining of CD68-positive interstitial macrophages (38). These findings support a role for interstitial macrophages in the pathogenesis of IPF. Furthermore, there is increasing evidence that macrophages can polarize along Th1 or Th2 pathways (39, 40). Our results showing that CCR4 is significantly expressed on macrophages would suggest that this might be true in pulmonary fibrosis. The expression of CCL17 in epithelial cells in bleomycin-treated lungs and IPF suggests that the epithelium has an important role in the recruitment of macrophages in IPF. Further indirect support for the role of CCL17 in macrophage recruitment during the pathogenesis of bleomycin-induced pulmonary fibrosis is the lack of a direct effect of CCL17 on fibroblast proliferation.

Previous work has shown that neutralization of CCL17 in a model of fulminant hepatic injury was associated with decreased numbers of CD4 lymphocytes and attenuation of the injury (41). Although we also found decreased numbers of CD4 lymphocytes the role of lymphocytes in bleomycin-induced pulmonary fibrosis is controversial (42). SCID mice develop fibrosis in similar fashion to wild-type mice (42). Interestingly there are increased numbers of eosinophils in areas of developing fibrosis in SCID mice (42). In contrast Schriber et al. (43) demonstrated attenuation of fibrosis in T cell-deficient nude mice as compared with euthymic mice, although these findings were in contrast to an earlier study showing similar fibrosis in nude and euthymic mice (44). Resistant BALB/c mice that were pretreated with cyclophosphamide were more susceptible to bleomycin than control mice, and this could be overcome with the administration of T cells from control mice (45). Depletion of CD4 or CD8 lymphocytes has been shown to attenuate bleomycin-induced pulmonary fibrosis (46). Interestingly, combined depletion of CD4 and CD8 lymphocytes has a greater than additive benefit over depletion of either subset alone (46). One of the reasons for the apparent disparities among these different studies may be the role of T regulatory cells. There is increasing evidence for the presence of regulatory T cells in both the CD4 and CD8 populations and selective depletion of one or more subsets may have differing effects (47).

In summary, we have shown that in the context of bleomycin-induced pulmonary fibrosis there is increased expression of CCL17, CCL22, and CCR4. CCR4 is highly expressed on macrophages. Depletion of CCL17 attenuated fibrosis and pulmonary inflammatory cell numbers. Furthermore, we have shown that CCL17 is localized to the epithelium in both the bleomycin model and human IPF lung tissue suggesting an important role for the epithelium in the pathogenesis of pulmonary fibrosis. It has recently been suggested that epithelial injury may have an important role in the pathogenesis of IPF (48, 49). Our findings support an integrated role for the epithelium, inflammatory cells, and Th2 type chemokines in the pathogenesis of pulmonary fibrosis. Attempts at switching the fibrotic phenotype toward a Th1 response may be more effective than targeting individual ligands or receptors.

References

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FIGURE 8. Photomicrograph showing immunolocalization of CCL17 from IPF lung tissue specimens. A, IPF lung section (magnification, ×312) stained with anti-CCL17 Abs demonstrating immunolocalization to epithelial cells. B, IPF lung section (magnification, ×312) stained with control Abs demonstrating the lack of nonspecific staining.


