Therapeutic Targeting of CC Ligand 21 or CC Chemokine Receptor 7 Abrogates Pulmonary Fibrosis Induced by the Adoptive Transfer of Human Pulmonary Fibroblasts to Immunodeficient Mice

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Idiopathic interstitial pneumonias (IIPs) are a collection of pulmonary fibrotic diseases of unknown etiology. CC chemokine receptor 7 (CCR7) is expressed in IIP biopsies and primary fibroblast lines, but its role in pulmonary fibrosis was not previously examined. To study the *in vivo* role of CCR7 in a novel model of pulmonary fibrosis, 1.0×10^6 primary fibroblasts grown from idiopathic pulmonary fibrosis/usual interstitial pneumonia, nonspecific interstitial pneumonia, or histologically normal biopsies were injected intravenously into C.B-17 severe combined immunodeficiency (SCID)/beige (bg) mice. At days 35 and 63 after idiopathic pulmonary fibrosis/usual interstitial pneumonia fibroblast injection, patchy interstitial fibrosis and increased hydroxyproline were present in the lungs of immunodeficient mice. Adoptively transferred nonspecific interstitial pneumonia fibroblasts caused a more diffuse interstitial fibrosis and increased hydroxyproline levels at both times, but injected normal human fibroblasts did not induce interstitial remodeling changes in C.B-17SCID/bg mice. Systemic therapeutic immunoneutralization of either human CCR7 or CC ligand 21, its ligand, significantly attenuated the pulmonary fibrosis in groups of C.B-17SCID/bg mice that received either type of IIP fibroblasts. Thus, the present study demonstrates that pulmonary fibrosis is initiated by the intravenous introduction of primary human fibroblast lines into immunodeficient mice, and this fibrotic response is dependent on the interaction between CC

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A persistently high mortality rate plagues idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP), the severest form of idiopathic interstitial pneumonia (IIP).¹ This is due to the failure of conventional immunomodulatory therapies, such as corticosteroids, azathioprine, and cyclophosphamide, to halt effectively the aggressive profibrotic and tissue repair processes in this disease.² Newly diagnosed IPF/UIP patients face respiratory failure and a plethora of complications including cardiovascular disease, lung cancer, and pulmonary embolism; most succumb to this disease within 3 to 5 years of diagnosis.³ With its growing prevalence worldwide,⁴ the need for novel therapies for IPF/UIP has become a major research focus, but a poor understanding of the etiopathogenesis of this disease has slowed progress in this regard.⁵ The aberrant parenchymal remodeling in IIP is characterized by the expansion of fibroblasts and myofibroblasts, and previous studies have shown that IPF/UIP fibroblasts have unique proliferation and synthetic properties relative to fibroblasts from other IIPs or normal lung tissues.^{6–9} These cells may arise from alterations in their microenvironment (due to dysregulated epithelial cell/mesenchymal cell interactions¹⁰) or be recruited out of the bone marrow (see reviews.^{11,12}). Regardless of the source of these cells, controlling their activity in the lung is paramount, and ongoing genomic and proteomic analyses of intact lung biop-

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sies^{13,14} and biopsy-derived fibroblasts¹⁵ have revealed putative targets to achieve this regulation.

Mouse models of pulmonary fibrosis have provided experimental paradigms with which to address abnormal tissue remodeling and scarring in the respiratory system.¹⁶ A number of approaches have been used to induce pulmonary fibrosis, and these include transgenic and gene transfer, radiation, inorganic irritants such as silica, and drugs promoting oxidant-induced inflammatory injury such as bleomycin.¹⁷ Of these models, the bleomycin model remains the most widely used because of its reproducibility and pathological similarity to human pulmonary fibrosis.¹⁷ Accordingly, the bleomycin model has been used to assess a number of targets of interest in IPF/UIP.^{13,14} Unfortunately, no animal model exists that fully recapitulates the clinico-pathological features of IPF/UIP, and debate still exists pertaining to the relative importance of ongoing inflammatory injury (the primary mode for inducing experimental fibrosis) to end-stage UIP.^{18,19}

Given this dilemma, the present study addressed an alternative strategy for inducing experimental pulmonary fibrosis. Mice that are genetically immunodeficient due to severe combined immunodeficiency (*scid*) gene mutation or recombinase-activating gene 1 (*rag-1*) knockout have been extensively used as *in vivo* hosts of adoptively transferred normal or diseased human cells. Herein, we report that the adoptive intravenous (i.v.) transfer of either IPF/UIP or nonspecific interstitial pneumonia (NSIP; another less severe form of IIP²⁰) but not normal fibroblasts into C.B-17 mice with the *scid-beige* (C.B-17SCID/bg) mutation initiated and maintained pulmonary fibrosis. Histological and biochemical evidence of fibrosis was first evident in both IIP fibroblast groups of C.B-17SCID/bg mice at day 35 and was prominent at day 63 after fibroblast injection. Cytokines and chemokines both seem to have major roles in the pathogenesis of IIP,²¹ and enzyme-linked immunosorbent assay (ELISA) analysis of whole-lung samples from C.B-17SCID/bg mice that received either IPF/UIP or NSIP fibroblasts revealed significant elevations in murine interleukin (IL)-13, CC ligand (CCL) 6, and CCL21 at day 63 compared with whole-lung levels measured at the earlier time point or in lung tissue from mice that did not receive fibroblasts. IL-13^{22,23} and CCL6²⁴ are mediators of pulmonary fibrosis, but the role of CCL21 in pulmonary remodeling events was unknown. Impetus to examine the role of CCL21 and CC chemokine receptor 7 (CCR7) in the pulmonary remodeling events precipitated by human IIP fibroblasts stemmed from our recent finding that CCR7 expression was increased in IIP biopsies,²⁵ and the migratory, synthetic, and proliferative properties of IIP fibroblasts are significantly enhanced by CCL21 (E.M.P. and C.M.H., unpublished data). In separate immunoneutralization studies, the targeting of either human CCL21 or CCR7 (the receptor for CCL21²⁶) from days 35 to 63 after IIP fibroblast injection into C.B-17SCID/bg mice significantly reduced all parameters of pulmonary fibrosis compared with groups of C.B-17SCID/bg mice receiving IIP fibroblasts and IgG. Together, these data highlight the creation of a new murine model of pulmonary fibrosis initiated and maintained by the i.v. introduction of IIP fibroblasts into C.B-17SCID/bg mice and demonstrate a novel role for

CCL21 and CCR7 in the maintenance of fibrosis in this model.

Materials and Methods

Mice

Female, ICR-*scid* (ICRSCID), C.B-17-*scid* (C.B-17SCID), and C.B-17-*scid-beige* (C.B-17SCID/bg) mice (6 to 8 weeks old) were purchased from Taconic Farms (Germantown, NY), and all SCID mice were housed in a gnotobiotic barrier facility at the University of Michigan Medical School. The first two groups of mice have the *scid* mutation leading to a lack of both T and B lymphocytes due to a V(D)J recombination defect, whereas C.B-17SCID/bg mice have two mutations: the first is the *scid* mutation, and the second is a *beige* mutation leading to a major defect in cytotoxic T-cell and macrophage function and a selective impairment in NK cell function. All mice had access to autoclaved water and pelleted mouse diet *ad libitum*. All procedures described below were performed in a sterile, laminar environment and were approved by an animal care and use committee at the University of Michigan Medical School.

Human Fibroblast Culture

A mixed cell population was obtained from mechanically dissociated IPF/UIP and NSIP surgical lung biopsies, and pure human fibroblast cultures were derived as previously described in detail.⁹ Normal lung fibroblasts were purified in the same manner from cell suspensions of normal margins associated with resected lung tumor tissue. In the present study, a total of 10 IPF/UIP, six NSIP, and four normal fibroblast lines were used after the fourth passage in the initial, model characterization, and therapeutic intervention studies described herein. An institutional review board at the University of Michigan Medical School approved this study.

Intravenous Introduction of Human Pulmonary Fibroblasts into SCID Mice

Single-cell preparations of IPF/UIP, NSIP, and normal fibroblasts were obtained after trypsinization of 150-cm² tissue culture flasks and labeled with PKH26 dye according to the manufacturer's directions (Sigma Co., St. Louis, MO). Each labeled fibroblast line was diluted to 1×10^6 cells/ml of phosphate-buffered saline (PBS), and 1 ml of this suspension was injected via a tail vein into groups of five SCID mice. Other groups of five SCID mice were injected intravenously with PKH26 and PBS labeling solution alone (ie, control group). Mice were euthanized by anesthesia overdose at days 7, 21, 35, 49, and 63 after the i.v. human pulmonary fibroblast transfer. Whole-lung tissue was dissected at these times for molecular, histological, biochemical, and/or proteomic analysis (see below).

Assessing the Role of CCL21 and CCR7 in C.B-17SCID/bg Mice after the Adoptive Transfer of Human Pulmonary Fibroblasts

To address the role of CCL21 and CCR7, its receptor, in the pulmonary remodeling response after the i.v. adoptive transfer of human fibroblasts, groups of C.B-17SCID/bg mice received IPF/UIP ($n = 35$ mice), NSIP ($n = 20$ mice), normal fibroblasts ($n = 15$ mice), or vehicle (ie, PBS) alone ($n = 15$ mice). Thirty-five days later, all groups of five C.B-17SCID/bg mice received mouse IgG, mouse anti-human CCL21 monoclonal antibody, or mouse anti-human CCR7 monoclonal antibody (all at $10 \mu\text{g/ml}$; R&D Systems, Minneapolis, MN) every other day from days 35 to 63. At day 63, all mice were euthanized by anesthesia overdose, and whole-lung tissue was dissected for molecular, histological, biochemical, and proteomic analysis (see below).

Molecular Analysis

Total RNA was isolated and cDNA generated from whole-lung samples as previously described in detail.²⁷ Changes in gene profiles for human and murine chemokine and chemokine receptors were analyzed in pooled samples ($n = 5$) using nonradioactive GEArray gene array membranes according to the manufacturer's instructions (SuperArray, Inc., Bethesda, MD), and signal intensities were determined as previously described in detail.²⁸ Individual whole-lung cDNA samples ($n \geq 5$) were analyzed for human CCR7, collagen I, cathepsin E, matrix metalloproteinase (MMP)-2, MMP-9, MMP-19, fibronectin, tissue inhibitors of metalloproteinases (TIMP)-1, and glyceraldehyde-3-phosphate dehydrogenase expression by real-time quantitative RT-PCR procedure using an 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) as previously described.²⁷

Histological Analysis

After anesthesia-induced euthanasia, the right lobes from each mouse were dissected, fully inflated with 10% formalin solution, and placed in fresh formalin for 24 hours. Standard histological techniques were used to paraffin-embed each lobe, and $5\text{-}\mu\text{m}$ sections were stained with hematoxylin and eosin and Mason trichrome for histological analysis. Additional unstained whole-lung tissue sections were analyzed via fluorescent microscopy.

Biochemical Analysis

Whole-left lung samples were homogenized in $1\times$ PBS and pelleted by centrifugation. The cell-free supernatants were removed for ELISA analysis, and the pellets were vacuum-dried and resuspended in 0.5 mol/L glacial acetic acid. The tissue was then processed for hydroxyproline concentration as previously described.²⁹

Proteomic Analysis

Murine IL-13, CCL6, CCL21, interferon- γ , IL-12, IL-4, CCL2, CCL7, CCL17, CCL3, CXC chemokine ligand (CXCL)13, tumor necrosis factor- α , CXCL10, CXCL9, and CXCL2 proteins were analyzed in $50 \mu\text{l}$ of cell-free supernatants from homogenized whole-lung samples using a standardized sandwich ELISA technique (R&D Systems) as previously described in detail.²⁷

Statistical Analysis

All results are expressed as mean $\pm$ SEM. One-way analysis of variance analysis and Tukey-Kramer or Dunnett's multiple comparisons tests were used to detect statistical differences between UIP, NSIP, normal, and control SCID mouse groups. Significance was set at $P < 0.05$.

Results

The Adoptive Intravenous Transfer of Either IPF/UIP or NSIP, but Not Normal, Pulmonary Fibroblasts Promoted Lung Histopathology and Remodeling in C.B-17SCID/bg Mice

Initial studies were undertaken to assess the impact of adoptively transferred normal and IIP fibroblasts on the lung architecture in various strains of SCID mice including ICRSCID, C.B-17SCID, and C.B-17SCID/bg ($n = 5$ per time point). These initial studies were undertaken using three IPF/UIP and two normal human fibroblast lines. All human fibroblast lines were labeled with PKH26 before injection into the SCID mouse groups thereby allowing for the detection of labeled cells in histological sections. At days 7 (Figure 1, B and C) and 21 (not shown) after injection, collections of human fibroblasts were detected in pulmonary blood vessels in each SCID group. However, the intensity of this marker diminishes after 21 days (information from the provided Sigma data sheet), and hence, we failed to detect PKH26 fluorescence in histological sections from each at day 35 after fibroblast injection (not shown). Other organs (ie, liver, spleen, and kidney) presumably contained human pulmonary fibroblasts, but we observed no evidence of gross macroscopic alterations to these organs. Because IIP is a lung-specific disease,³⁰ which does not exhibit fibrogenesis in any other organ, a detailed histological analysis of other organs was not undertaken in the present study.

Examination of whole-lung sections in groups of SCID mice at later time points revealed that marked lung remodeling was only present in C.B-17SCID/bg mice. Specifically, no fibroproliferation was observed at day 49 after the introduction of IPF/UIP fibroblasts into ICRSCID mice ($n = 5$ mice; not shown). Likewise, the i.v. adoptive transfer of IPF/UIP fibroblasts into C.B-17SCID mice failed to elicit histologically evident pulmonary remodeling at days 7 ($n = 5$ mice), 21 ($n =$

Figure 1. Representative whole-lung tissues sections from C.B-17SCID/bg mice that received PBS + PKH26 (**A**) or IPF/UIP fibroblasts + PBS + PKH26 (**B** and **C**). The presence of fluorescently labeled human fibroblasts in the lung was observed in C.B-17SCID/bg mice that received human IPF/UIP fibroblasts 7 days previously (**B** and **C**). The clumped human fibroblasts were typically detected in the walls of pulmonary blood vessels (**B** and **C**). Original magnifications: $\times 100$ (**A**), $\times 200$ (**B**), and $\times 400$ (**C**).

10 mice), 35 ($n = 5$ mice), or 49 ($n = 5$ mice) after the i.v. injection of IPF/UIP fibroblasts.

Given the presence of marked pulmonary histopathology in the C.B-17SCID/bg group, all subsequent studies described below involved the adoptive transfer of normal and IIP fibroblasts into C.B-17SCID/bg mice. In the model characterization study, four IPF/UIP, four NSIP, and one normal fibroblast lines were adoptively transferred into separate groups of C.B-17SCID/bg mice, and pulmonary histopathological, genomic, and proteomic alterations were analyzed at days 35 and 63 after fibroblast transfer.

Little or no pulmonary histopathology was observed in C.B-17SCID/bg mice that received normal pulmonary fibroblasts (Figure 2A). However, C.B-17SCID/bg mice exhibited significant pulmonary histopathology, which was evident at day 35 after the i.v. injection of either NSIP (Figure 2B) or IPF/UIP (Figure 2C) fibroblast lines. The pulmonary histopathology in C.B-17SCID/bg mice following NSIP or IPF/UIP pulmonary fibroblasts was characterized by disruption of the alveolar space, apparent fibroproliferation, and the presence of eosinophilic granulocytes (Figure 2, B and C). Mason trichrome staining of histological tissue sections from the lungs of C.B-17SCID/bg mice at day 35 after the adoptive transfer of normal (Figure 2D), NSIP (Figure 2E), or IPF/UIP (Figure

2F) human pulmonary fibroblasts revealed the presence of extracellular matrix (stained light blue) in remodeled areas in C.B-17SCID/bg groups that received the IIP but not normal fibroblasts.

Later analysis of histological sections from C.B-17SCID/bg groups revealed major differences in the extent and appearance of the pulmonary remodeling precipitated by the introduction of IPF/UIP or NSIP fibroblasts. The lungs of C.B-17SCID/bg mice that received IPF/UIP fibroblasts 63 days previously exhibited a heterogeneous appearance with areas of relatively normal-appearing lung tissue (Figure 3A) adjacent to areas of severe interstitial disruption and remodeling (Figure 3B). In addition, foci of human fibroblasts were apparent in the lungs of C.B-17SCID/bg mice that received IPF/UIP fibroblasts (Figure 3B, inset), but these foci were detected in blood vessels and not in interstitial areas as in clinical UIP.¹ The histological pattern in whole-lung tissue sections from C.B-17SCID/bg mice that received NSIP fibroblasts 63 days previously was characterized by interstitial thickening and overtly fibrotic areas, and the remodeling in these mice appeared to involve most of the lung (Figure 3, C and D). Together, these data showed that the introduction of human IIP fibroblasts into C.B-17SCID/bg mice caused fibrotic lesions in these mice.

Figure 2. Representative hematoxylin and eosin-stained histological sections from C.B-17SCID/bg mice that received normal (A), NSIP (B), or IPF/UIP (C) fibroblasts. Representative Mason trichrome-stained histological sections from C.B-17SCID/bg mice that received normal (D), NSIP (E), or IPF/UIP (F) fibroblasts are shown. Lung samples were removed at day 35 after the adoptive i.v. transfer of human pulmonary fibroblasts into C.B-17SCID/bg mice. Original magnification, $\times 200$ (A, C–F) or $\times 400$ (B).

Hydroxyproline Levels Were Significantly Altered in a Temporally Dependent Manner after the Introduction of IIP Fibroblasts into C.B-17SCID/bg Mice

Hydroxyproline is a commonly used marker of *de novo* collagen synthesis in experimental models involving pulmonary remodeling.³¹ In the present study, hydroxyproline levels were measured in whole-lung samples from C.B-17SCID/bg mice that had received no fibroblasts or normal, NSIP, or IPF/UIP human pulmonary fibroblasts either 35 or 63 days previously. As shown in Figure 4, hydroxyproline levels were unchanged at these time points after the introduction of normal fibroblasts. The levels of hydroxyproline in these C.B-17SCID/bg groups were 4.7 ± 0.3 and 5.6 ± 0.5 $\mu\text{g}/\text{mg}$ protein at days 35 and 63, respectively, and these hydroxyproline levels were similar to those detected in C.B-17SCID/bg mouse groups that did not receive human fibroblasts (4.5 ± 1.3 $\mu\text{g}/\text{mg}$ protein). However, the lungs of mice contained greater amounts of hydroxyproline at day 35 after intravenously transferred NSIP (11.4 ± 3.7 $\mu\text{g}/\text{mg}$ protein) or IPF/UIP (8.2 ± 2 $\mu\text{g}/\text{mg}$ protein) fibroblasts compared with the normal fibroblast group. In addition, hydroxyproline levels were further increased 3- and 2.5-fold in the NSIP (31 ± 11 $\mu\text{g}/\text{mg}$ protein) and IPF/UIP (22 ± 4 $\mu\text{g}/\text{mg}$ protein) fibroblast groups, respectively, at day 63 after i.v. adoptive transfer (Figure 4). At the day-63 time point, the increase in hydroxyproline levels in the C.B-17SCID/bg groups that received either NSIP or IPF/UIP human fibroblasts reached statistical significance compared with the C.B-17SCID/bg group that received nor-

mal fibroblasts. Thus, the intravenous injection of human IIP fibroblasts enhanced hydroxyproline levels at day 35 and, most demonstrably, at day 63 after adoptive transfer.

Whole-Lung Cytokine Analysis Showed That Murine IL-13, CCL6, and CCL21 Were Significantly Elevated in the Lungs of C.B-17SCID/bg Mice That Received IIP Fibroblasts

Whole-lung ELISA analysis of several cytokines, CC ligand, and CXC ligand chemokines at days 35 and 63 after the i.v. adoptive transfer of human normal or IIP fibroblasts revealed a number of statistically significant changes in murine IL-13, CCL6, and CCL21 (Figure 5). Although the whole-lung levels of IL-13 in the C.B-17SCID/bg group that received normal fibroblasts were below the level of ELISA detection, whole-lung IL-13 levels were significantly greater in the C.B-17SCID/bg group that received either NSIP or IPF/UIP fibroblasts compared with the C.B-17SCID/bg group that received normal fibroblasts 35 and/or 63 days previously (Figure 5, top). In addition, significantly more IL-13 was detected in the lungs of C.B-17SCID/bg mice that received IPF/UIP fibroblasts at day 63 versus 35 after adoptive transfer of fibroblasts. At days 63 after fibroblast injection, whole-lung CCL6 levels were significantly greater in the C.B-17SCID/bg groups that received either IPF/UIP or NSIP fibroblasts compared with the C.B-17SCID/bg group that received

Figure 3. Representative Mason trichrome-stained histological sections from C.B-17SCID/bg mice that received IPF/UIP (**A** and **B**) or NSIP (**C** and **D**) fibroblasts 35 or 63 days previously. Normal-appearing areas (**A**), intensely fibrotic areas (**B**), and foci of human fibroblasts (observed at day 35 after fibroblast injection; inset to **B**) were apparent in whole-lung histological sections from C.B-17SCID/bg mice that received IPF/UIP fibroblasts. In contrast, whole-lung histological sections from C.B-17SCID/bg mice that received NSIP showed a more generalized involvement of the lung tissue examined with varying degrees of fibrosis and remodeling (**C** and **D**). Lung samples were removed at days 35 or 63 after the adoptive i.v. transfer of human fibroblasts into C.B-17SCID/bg mice. Original magnification, $\times 200$.

normal fibroblasts (Figure 5, middle). Significantly greater CCL6 was detected in the lungs of C.B-17SCID/bg mice that received IPF/UIP or NSIP fibroblasts at day 63 versus 35 after adoptive transfer of

Figure 4. Whole-lung hydroxyproline levels in C.B-17SCID/bg mice that received normal, NSIP, or IPF/UIP fibroblasts. Lung samples were removed at days 35 and 63 after the adoptive i.v. transfer of human fibroblasts into C.B-17SCID/bg mice. Data shown are mean $\pm$ SEM. * $P \leq 0.05$ compared with hydroxyproline levels measured at day 63 after adoptive transfer of normal fibroblasts into C.B-17SCID/bg mice.

fibroblasts (Figure 5, middle). The only other murine CC ligand that was altered by the introduction of human fibroblasts into C.B-17SCID/bg mice was CCL21. This chemokine was significantly greater in the NSIP and IPF/UIP fibroblast C.B-17SCID/bg groups at day 63 compared with the normal fibroblast C.B-17SCID/bg groups at day 63. In addition, whole-lung murine CCL21 levels were significantly elevated in IPF/UIP fibroblast groups at day 63 after fibroblast transfer compared with the NSIP fibroblast group at the same time (Figure 5, bottom). Finally, significantly higher levels of CCL21 were present in whole-lung samples from the IIP fibroblasts C.B-17SCID/bg group at day 63 after adoptive transfer compared with the IIP fibroblasts C.B-17SCID/bg groups at day 35 after adoptive transfer. Thus, taken together, these data suggested that the presence of IIP fibroblasts, but not normal fibroblasts, in C.B-17SCID/bg mice significantly altered the whole-lung levels of murine cytokines and chemokines with established (ie, IL-13^{22,23} and CCL6²⁴) and putative (ie, CCL21) profibrotic roles in the lung.

Figure 5. Whole-lung murine IL-13, CCL6, and CCL21 levels in C.B-17SCID/bg mice that received normal, NSIP, or IPF/UIP fibroblasts. Lung samples were removed at days 35 and 63 after the adoptive i.v. transfer of human fibroblasts into C.B-17SCID/bg mice, and all soluble proteins were measured by specific ELISA. Data shown are mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with appropriate the C.B-17SCID/bg group that received normal fibroblasts. † $P \leq 0.01$, †† $P \leq 0.001$ compared with whole-lung cytokine and chemokine levels at the day 35 time point in the C.B-17SCID/bg groups with either IPF/UIP or NSIP fibroblasts. The whole-lung cytokine and chemokine levels in control C.B-17SCID/bg group that did not receive fibroblasts were as follows: IL-13, 0.18 ± 0.014 ng/mg protein; CCL6, 0.37 ± 0.05 ng/mg protein; and CCL21, 2.0 ± 0.1 ng/mg protein.

Human CCR7 and CCL21 Gene Transcripts Were Present in the Lungs of C.B-17SCID/bg Mice That Received Human Pulmonary Fibroblasts 35 Days Previously

The changes in whole-lung levels of murine CCL21 in C.B-17SCID/bg mice that received either NSIP or IPF/UIP

Table 1. SuperArray Analysis of Human CCR7 and CCL21 and TaqMan Analysis of Human CCR7 in Whole-Lung Samples from C.B-17SCID/bg Mice at Day 35 after i.v. Human Fibroblast Injection

Day 35 after injection	Fibroblast source	
	CCR7	CCL21
Intensity of signal for gene		
IPF/UIP	47	16
NSIP	2	n.d.
Normal	1	5
(2 ^{-ΔΔCt})		
IPF/UIP	21	
NSIP	12 $\pm$ 6	
Normal	7 $\pm$ 1	

n.d., not detected.

fibroblasts were intriguing in light of previous studies demonstrating the important remodeling role of this CC ligand in the kidney³² and liver.³³ This prompted further analysis of this CC ligand and its receptor, CCR7, during the pulmonary remodeling responses elicited by human IIP fibroblasts. Human transcripts for CCR7 and CCL21 were detected by SuperArray gene analysis, and among the three C.B-17SCID/bg groups, the greatest transcript expression for CCR7 and CCL21 was present in lung samples from the IPF/UIP fibroblast group (Table 1). Furthermore, the presence of CCR7 was confirmed by TaqMan analysis, and again the IPF/UIP group exhibited the highest CCR7 transcript expression among the C.B-17SCID/bg groups (Table 1). SuperArray and TaqMan analysis of murine CCR7 and CCL21 also confirmed the presence of these transcript products in all three C.B-17SCID/bg groups that received human fibroblasts, and the highest levels of both transcripts were present in the C.B-17SCID/bg groups that received IPF/UIP fibroblasts (not shown). Thus, the i.v. adoptive transfer of normal and IIP fibroblasts resulted in the presence of human gene transcripts for CCR7 and CCL21.

Quantitative TaqMan PCR Analysis of Murine Extracellular Matrix-Associated Genes after the Adoptive Transfer of Human Fibroblasts into C.B-17SCID/bg Mice

Lung alterations in murine extracellular matrix-associated genes were analyzed using quantitative PCR analysis. As shown in Figure 6, top, the expression of collagen 1, cathepsin E, MMP-19, and TIMP-1 was present in C.B-17SCID/bg mice that received normal, NSIP, and IPF/UIP human fibroblasts 63 days previously. Most importantly, when transcript levels for these genes in fibroblast-challenged mice were compared with transcript levels in control C.B-17SCID/bg mice, the increases in collagen 1 and cathepsin transcript expression in C.B-17SCID/bg mice that received either type of IIP fibroblast and MMP-19 and TIMP-1 transcript expression in mice that received IPF/UIP fibroblasts reached statistical significance when compared with the transcript increases for these genes in C.B-17SCID/bg mice that received normal

Figure 6. Quantitative TaqMan PCR analysis of extracellular matrix-associated genes, collagen 1, cathepsin E, MMP-19, TIMP-1, MMP-2, MMP-9, and fibronectin in C.B-17SCID/bg mice that received normal, NSIP, or IPF/UIP fibroblasts. Changes in gene expression are expressed as mean $\pm$ SEM of the fold increase in transcript expression above a group of C.B-17SCID/bg mice that received PBS and PKH26 alone. * $P \leq 0.05$, ** $P \leq 0.01$ compared with the C.B-17SCID/bg group that received normal fibroblasts.

human fibroblasts (Figure 6, top). Other extracellular matrix genes were analyzed by TaqMan, and the presence of MMP-2, MMP-9, and fibronectin was confirmed. No significant differences in levels of these transcripts were observed among the groups of C.B-17SCID/bg mice, but the greatest increase in MMP-2 and fibronectin was observed in the IPF/UIP C.B-17SCID/bg group, whereas the smallest increase in MMP-9 was observed in this same C.B-17SCID/bg group (Figure 6, bottom). Thus, these data showed that transcript levels for murine extracellular matrix-associated genes were altered by the presence of human fibroblasts in C.B-17SCID/bg mice.

Immunoneutralization of Human CCR7 or Human CCL21 Abrogated Pulmonary Remodeling in C.B-17SCID/bg Mice That Received Human IIP Fibroblasts

In the next series of experiments, the roles of human CCR7 and CCL21 were assessed in C.B-17SCID/bg mice that received human normal ($n = 1$ line) or IIP fibroblasts ($n = 3$ IPF/UIP and $n = 2$ NSIP lines). Although attempts to mea-

sure human CCL21 in whole-lung samples were unsuccessful, presumably because of the presence of this CC ligand at levels below the level of ELISA detection (not shown), previous studies have shown that both mouse and human CCL21 can promote cellular calcium flux via human CCR7.³⁴ In addition, the immunoneutralization of mouse CCL21 using a polyclonal antibody has been shown to abrogate the migration of human dendritic cells and the priming of human T cells in a humanized model of house dust mite-induced allergic airway disease.³⁵ Given the reported cross-reactivity of mouse and human CCL21, a therapeutic protocol was implemented in which either human CCL21 or human CCR7 was targeted by monoclonal antibody administration from days 35 to 63 after the adoptive transfer of fibroblasts. A representative histological survey of whole-lung tissues at day 63 in the three C.B-17SCID/bg groups with the three treatment modalities tested is shown in Figure 7. In the normal fibroblast C.B-17SCID/bg group, no evidence of interstitial pulmonary remodeling was evident in any of the treatment groups (IgG treatment, Figure 7A; anti-CCL21 antibody treatment, Figure 7B; and anti-CCR7 antibody treatment, Figure 7C). However, the vascular accumulation of normal fibroblasts was histologically apparent in these C.B-17SCID/bg groups, and an example is shown in Figure 7B. None of the treatments altered this feature in C.B-17SCID/bg mice that received normal fibroblasts. Interstitial remodeling was apparent in the NSIP fibroblast C.B-17SCID/bg group that received IgG (Figure 7D), but this remodeling response was abrogated by either anti-CCL21 antibody (Figure 7E) or anti-CCR7 antibody (Figure 7F) administration from days 35 to 63 after adoptive transfer. Likewise, the interstitial remodeling apparent in the IgG C.B-17SCID/bg group that received IPF/UIP fibroblasts (Figure 7G) was absent in groups of C.B-17SCID/bg mice that received anti-CCL21 antibody (Figure 7H) or anti-CCR7 antibody (Figure 7I) administration from days 35 to 63 after adoptive transfer IPF/UIP fibroblasts. Thus, therapeutic targeting of either CCL21 or CCR7 abrogated histological evidence of interstitial remodeling in C.B-17SCID/bg mice that received IIP fibroblasts.

Quantitative TaqMan PCR Analysis of Murine Extracellular Matrix-Associated Genes after the Therapeutic Targeting of Either Human CCL21 or CCR7 in C.B-17SCID/bg Mice That Received Human Normal and IIP Fibroblasts

Quantitative TaqMan analysis of the antibody treatment groups also confirmed that the anti-CCL21 and anti-CCR7 treatments also significantly altered the transcript levels of MMP-2 and MMP-19. Transcript levels for these MMPs in antibody-treated, control C.B-17SCID/bg mice were compared with the levels of these transcripts in antibody-treated, fibroblast-challenged C.B-17SCID/bg mice. Anti-CCL21 antibody treatment significantly reduced the fold increase in MMP-2 transcript levels in C.B-17SCID/bg groups that received normal fibroblasts compared with the fold increase in the transcript levels in C.B-17SCID/bg groups that received IgG (Figure 8, top). The anti-CCR7

Figure 7. Representative Mason trichrome-stained histological sections from C.B-17SCID/bg mice that received normal (A–C), NSIP (D–F), or IPF/UIP (G–I) fibroblasts. No interstitial remodeling was apparent in C.B-17SCID/bg mice that received normal fibroblasts, but vascular anomalies were observed in this group (B), and the IgG (A), anti-CCL21 monoclonal antibody (B), and anti-CCR7 monoclonal antibody (C) therapies did not alter the lung histological appearance in this group. Pulmonary remodeling was apparent in C.B-17SCID/bg mice that received NSIP fibroblasts, and this pattern was not altered by IgG (D), whereas the anti-CCL21 antibody (E) or anti-CCR7 antibody (F) therapies markedly reduced the interstitial remodeling in whole-lung samples. Interstitial pulmonary fibrosis was apparent in C.B-17SCID/bg mice that received IPF/UIP fibroblasts, and this pattern was not altered by IgG (G), whereas the anti-CCL21 antibody (H) or anti-CCR7 antibody (I) therapies markedly reduced the interstitial remodeling in whole-lung samples. Monoclonal antibody therapies began at day 35 and continued to day 63, and lung samples were removed at day 63 after the adoptive i.v. transfer of human fibroblasts into C.B-17SCID/bg mice. Original magnification, $\times 400$.

antibody treatment significantly reduced the fold increase in MMP-2 transcript levels compared with the appropriate IgG group. Quantitative TaqMan analysis of MMP-19 revealed that anti-CCR7 significantly increased the fold change in the C.B-17SCID/bg group that received normal fibroblasts, whereas both antibody treatments significantly increased the fold change in this transcript compared with the appropriate IgG group (Figure 8, bottom). Thus, the antibody treatments used in C.B-17SCID/bg mice challenged with human fibroblasts markedly altered transcript expression for murine extracellular matrix-associated genes.

Immunoneutralization of Human CCR7 or Human CCL21 Significantly Reduced Whole-Lung Hydroxyproline Levels in C.B-17SCID/bg Mice That Received Human IPF Fibroblasts

Hydroxyproline levels in whole-lung samples from IgG-, anti-CCL21-, or anti-CCR7-treated control C.B-

17SCID/bg mice (ie, mice that did not receive human fibroblasts) and groups of treated mice that received normal, NSIP, or IPF/UIP fibroblasts are shown in Figure 9. Hydroxyproline levels were increased in whole-lung samples from C.B-17SCID/bg mice that received normal fibroblasts and IgG, but neither antibody treatment altered these levels (Figure 9). In the NSIP fibroblast C.B-17SCID/bg groups, hydroxyproline levels were significantly increased above those levels measured in the control C.B-17SCID/bg group, and the anti-CCL21 antibody and anti-CCR7 antibody therapies significantly reduced hydroxyproline levels by 52 ± 6.7 and $51 \pm 5.6\%$, respectively, compared with the IgG treatment group (Figure 9). In the IPF/UIP fibroblast C.B-17SCID/bg group that received IgG, hydroxyproline levels were again significantly increased above those levels measured in the control C.B-17SCID/bg group. In addition, hydroxyproline levels in IPF/UIP fibroblast challenged mice were reduced by 66 ± 7.2 and $59 \pm 7.1\%$ in the anti-CCL21 and anti-CCR7 antibody treatment groups, respectively,

Figure 8. Quantitative TaqMan PCR analysis of extracellular matrix-associated genes MMP-2 (top) and MMP-19 (bottom) in C.B-17SCID/bg mice that received normal, NSIP, or IPF/UIP fibroblasts. Changes in gene expression are expressed as mean $\pm$ SEM of the fold increase in transcript expression above a group of C.B-17SCID/bg mice that received PBS, PKH26, and one of IgG, anti-CCL21 antibody, and anti-CCR7 antibody. * $P \leq 0.05$, *** $P \leq 0.001$ compared with the appropriate C.B-17SCID/bg group that received human fibroblasts and IgG treatment.

compared with the IgG-treated C.B-17SCID/bg IPF/UIP fibroblast group (Figure 9). Thus, these data confirmed that the targeting of either CCL21 or CCR7 markedly and significantly reduced pulmonary remodeling precipitated by the adoptive transfer of NSIP or IPF/UIP fibroblasts in C.B-17SCID/bg mice.

Whole-Lung Cytokine Analysis Showed That Murine CCL21 Levels Were Significantly Reduced in the Lungs of C.B-17SCID/bg Mice That Received IPF/UIP Fibroblasts and Anti-CCR7 Antibody Therapy

Because whole-lung levels of murine IL-13, CCL6, and CCL21 were altered and/or increased by the presence of NSIP and IPF/UIP fibroblasts in C.B-17SCID/bg mice, the effects of the IgG and monoclonal antibody treatments on these mediators at day 63 were assessed. As shown in Figure 10, the anti-CCR7 and anti-CCL21 antibody therapies did not affect whole-lung levels of IL-13 or CCL6 in any of the C.B-17SCID/bg fibroblast groups. However,

Figure 9. Whole-lung hydroxyproline levels in C.B-17SCID/bg mice that received no fibroblasts (ie, control) or received normal, NSIP, or IPF/UIP fibroblasts. All groups of mice received either IgG or monoclonal antibody therapy. IgG, anti-CCL21, and anti-CCR7 monoclonal antibody therapies began in separate groups of C.B-17SCID/bg mice at day 35 and continued to day 63. Lung samples were removed at day 63 after the adoptive i.v. transfer of human fibroblasts into C.B-17SCID/bg mice. Data shown are mean $\pm$ SEM. * $P \leq 0.05$ compared with the control C.B-17SCID/bg group, which did not receive any human fibroblasts; $\tau\tau\tau P \leq 0.001$ compared with the appropriate C.B-17SCID/bg group that received human fibroblasts and IgG treatment; * $P \leq 0.05$ compared with the appropriate C.B-17SCID/bg group that received human fibroblasts and IgG treatment.

the anti-CCR7 antibody therapy significantly reduced whole-lung levels of murine CCL21 in the C.B-17SCID/bg group that received IPF/UIP fibroblasts compared with the C.B-17SCID/bg group that received IPF/UIP fibroblasts + IgG (Figure 10). Thus, the therapeutic targeting of CCR7 reduced murine CCL21 levels but did not affect the levels of the other two mouse mediators found to be elevated in the IIP fibroblast models.

Discussion

The present study addressed the following two questions: Do adoptively transferred human pulmonary fibroblasts remodel the lung architecture in SCID mice, and what role do CCL21 and CCR7 exert in the remodeling response precipitated by the adoptive transfer of human fibroblasts? The response to the first question was affirmative because the i.v. adoptive transfer of either 1×10^6 IPF/UIP or NSIP fibroblasts into mice C.B-17SCID/bg mice lacking both adaptive and innate immune features caused fibrosis, which was confirmed using histological, molecular, and biochemical analyses. This fibrosis was abrogated by the therapeutic administration of monoclonal antibodies directed against either human CCL21 or CCR7, thereby demonstrating a major role for this ligand and its receptor in pulmonary fibrosis similar to their roles in renal³² and liver³³ fibrosis. Although murine equivalents of known and putative profibrotic mediators were increased in C.B-17SCID/bg mice that had received either IPF/UIP or NSIP fibroblasts, the role of these mediators in the fibrotic process is questionable because their levels were unchanged in many groups of C.B-17SCID/bg mice, which showed a significant decrease in lung remodeling. Thus, the present study provides evidence that the adoptive transfer of IIP fibroblasts promotes fibrosis in

Figure 10. Whole-lung murine IL-13, CCL6, and CCL21 levels in C.B-17SCID/bg mice that received normal, NSIP, or IPF/UIP fibroblasts and IgG or monoclonal antibody therapies. Anti-CCL21 and anti-CCR7 monoclonal antibody therapies began at day 35 and continued to day 63. Lung samples were removed at day 63 after the adoptive i.v. transfer of human fibroblasts into C.B-17SCID/bg mice. Specific ELISA was used to measure all soluble proteins. Data shown are mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$ compared with indicated protein levels measured in whole-lung samples from C.B-17SCID/bg mice that received human fibroblasts.

C.B-17SCID/bg mice and identifies a novel therapeutic target in IIP.

The modeling of clinical pulmonary fibrosis remains a major challenge in the laboratory.¹⁷ Although bleomycin sulfate is the agent of choice in the induction of experimental fibrosis, bleomycin-induced pulmonary fibrosis has been criticized as a less than ideal model of IPF/UIP.¹⁷ Recognizing that newer models of pulmonary fibrosis should incorporate as many of the features of clinical IIP as possible, the present study capitalized on the observation that the fibroblast is primarily responsible for the profound and often lethal remodeling in these diseases.⁴ The adoptive transfer of either IPF/UIP or NSIP fibroblasts initiated interstitial remodeling and histologically evident fibrosis was observed at day 35, but not

earlier, after the i.v. adoptive transfer of these lines. The delay in appearance of fibrosis is not readily explainable, but these findings are consistent with previous studies in which the adoptive transfer of human endothelial cells into C.B-17SCID/bg mice required approximately 30 to 40 days before distinct blood vessels were apparent.³⁶ Thus, the present study highlights that a clinically relevant model of pulmonary fibrosis can be initiated in immunodeficient mice with the adoptive transfer of human pulmonary fibroblasts.

The obvious benefit of the IPF/UIP and NSIP fibroblast C.B-17SCID/bg models described herein is their utility in the testing of novel therapeutics for these diseases. The present study addressed the roles of human CCL21 and CCR7 because we have observed that both are prominently expressed in IIP biopsies²⁵ and cultured IIP fibroblasts (E.M.P. and C.M.H., unpublished observations). Our present hypothesis is that the therapeutic effect of anti-human CCL21 and anti-human CCR7 antibody treatments relates to their negations of the pro-proliferative effect of CCL21 on IPF/UIP and NSIP fibroblasts (E.M.P. and C.M.H., unpublished observations). Although it is unlikely that the monoclonal antibodies used herein affected the migration of i.v.-injected fibroblasts because the antibody treatments were delayed until a time point when fibrosis was histologically apparent in C.B-17SCID/bg mice, it is conceivable that this therapeutic approach, if used in other models of pulmonary fibrosis, may have an effect on the recruitment of fibrocytes into the lung. CCR7 is prominently expressed on fibrocytes,^{37,38} and CCL21 promotes their recruitment into various tissue sites.³⁷ Studies are presently underway to address the role of CCR7-positive fibrocytes in bleomycin-induced pulmonary fibrosis using CCR7 wild-type and gene-deficient mice.

The precise contribution of the adoptively transferred human IIP fibroblasts and the mouse-associated fibrotic components (ie, cells and mediators) to the overall pulmonary remodeling response observed in C.B-17SCID/bg mice remains to be determined. In the present study, it would seem that there was an interaction between the transferred human fibroblasts and mouse components as evidenced by the dynamic changes in murine extracellular matrix-associated transcripts and murine-soluble profibrotic proteins in the lungs of C.B-17SCID/bg mice that most notably received either of the two IIP fibroblast types. Although the relative importance of the soluble murine proteins detected in the C.B-17SCID/bg fibrotic response is questionable at this point, the alterations in cathepsin E, MMPs, extracellular matrix components (ie, collagen and fibronectin), and the inhibitors of MMPs in the IIP fibroblast C.B-17SCID/bg groups relative to the normal fibroblast C.B-17SCID/bg group may indicate that the mouse-associated fibrotic components were actively involved in the remodeling response. Quantitative TaqMan PCR confirmed that collagen 1, cathepsin E, MMP-19, and TIMP-1 transcript levels were significantly increased in the IIP fibroblast C.B-17SCID/bg groups relative to changes in transcript levels for these genes in C.B-17SCID/bg mice that received normal fibroblasts. These transcript changes are relevant

to clinical pulmonary fibrosis because cathepsins,^{39,40} MMPs and TIMPs (reviewed in Ref. ⁴¹), and extracellular matrix^{42,43} are increased in the more severe forms of IIP such as IPF/UIP and NSIP. MMP-19 was increased most notably in C.B-17SCID/bg mice that received IPF/UIP fibroblasts, and although this proteinase seems to have a major role in dermal wound healing responses (commentary by Mauch⁴⁴), its role in pulmonary fibrosis is unknown. Almost without exception, the anti-CCL21 and anti-CCR7 antibody therapies reduced all of the above-mentioned murine extracellular matrix-associated gene products analyzed using quantitative TaqMan PCR. The one exception was MMP-19, and further study of the relative importance of MMP-19 in the pulmonary fibrotic response evoked after the adoptive transfer of human IIP fibroblasts is warranted in light of this and our other findings.

In summary, the present study confirms that pulmonary fibrosis can be transferred to C.B-17SCID/bg mice after the i.v. adoptive transfer of either IPF/UIP or NSIP primary fibroblast lines. The utility of this model in the testing of novel therapeutic strategies was demonstrated, and our findings raise the possibility that the CCL21-CCR7 interaction may be an important target in clinical NSIP and IPF/UIP.

References

1. American Thoracic Society/European Respiratory Society (ATS/ERS) international multidisciplinary consensus classification of idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2002, 165:277–304
2. Antoniou KM, Pataka A, Bouros D, Siafakas NM: Pathogenetic pathways and novel pharmacotherapeutic targets in idiopathic pulmonary fibrosis. *Pulm Pharmacol Ther* 2006 [Epub ahead of Print]
3. Strieter RM: Pathogenesis and natural history of usual interstitial pneumonia: the whole story or the last chapter of a long novel. *Chest* 2005, 128:526S–532S
4. Zisman DA, Keane MP, Belperio JA, Strieter RM, Lynch III JP: Pulmonary fibrosis. *Methods Mol Med* 2005, 117:3–44
5. Swigris JJ, Kuschner WG, Kelsey JL, Gould MK: Idiopathic pulmonary fibrosis: challenges and opportunities for the clinician and investigator. *Chest* 2005, 127:275–283
6. Keane MP, Arenberg DA, Lynch JP III, Whyte RI, Iannettoni MD, Burdick MD, Wilke CA, Morris SB, Glass MC, DiGiorgine B, Kunkel SL, Strieter RM: The CXC chemokines, IL-8 and IP-10, regulate angiogenic activity in idiopathic pulmonary fibrosis. *J Immunol* 1997, 159:1437–1443
7. Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A: Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *Am J Respir Cell Mol Biol* 2001, 24:591–598
8. White ES, Atrasz RG, Hu B, Phan SH, Stambolic V, Mak TW, Hogaboam CM, Flaherty KR, Martinez FJ, Kontos CD, Toews GB: Negative regulation of myofibroblast differentiation by PTEN (phosphatase and tensin homolog deleted on chromosome 10). *Am J Respir Crit Care Med* 2006, 173:112–121
9. Jakubzik C, Choi ES, Carpenter KJ, Kunkel SL, Evanoff H, Martinez FJ, Flaherty KR, Toews GB, Colby TV, Travis WD, Joshi BH, Puri RK, Hogaboam CM: Human pulmonary fibroblasts exhibit altered interleukin-4 and interleukin-13 receptor subunit expression in idiopathic interstitial pneumonia. *Am J Pathol* 2004, 164:1989–2001
10. Selman M, Thannickal VJ, Pardo A, Zisman DA, Martinez FJ, Lynch III JP: Idiopathic pulmonary fibrosis: pathogenesis and therapeutic approaches. *Drugs* 2004, 64:405–430
11. Quan TE, Cowper SE, Bucala R: The role of circulating fibrocytes in fibrosis. *Curr Rheumatol Rep* 2006, 8:145–150
12. Lama VN, Phan SH: The extrapulmonary origin of fibroblasts: stem/progenitor cells and beyond. *Proc Am Thorac Soc* 2006, 3:373–376
13. Kaminski N, Allard JD, Pittet JF, Zuo F, Griffiths MJ, Morris D, Huang X, Sheppard D, Heller RA: Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. *Proc Natl Acad Sci USA* 2000, 97:1778–1783
14. Pardo A, Gibson K, Cisneros J, Richards TJ, Yang Y, Becerril C, Yousem S, Herrera I, Ruiz V, Selman M, Kaminski N: Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Med* 2005, 2:e251
15. Choi ES, Jakubzik C, Carpenter KJ, Kunkel SL, Evanoff H, Martinez FJ, Flaherty KR, Toews GB, Colby TV, Kazerooni EA, Gross BH, Travis WD, Hogaboam CM: Enhanced monocyte chemoattractant protein-3/CC chemokine ligand-7 in usual interstitial pneumonia. *Am J Respir Crit Care Med* 2004, 170:508–515
16. Gharaee-Kermani M, Ullenbruch M, Phan SH: Animal models of pulmonary fibrosis. *Methods Mol Med* 2005, 117:251–259
17. Chua F, Gaudie J, Laurent GJ: Pulmonary fibrosis: searching for model answers. *Am J Respir Cell Mol Biol* 2005, 33:9–13
18. Gaudie J: Pro: inflammatory mechanisms are a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2002, 165:1205–1206
19. Strieter RM: Con: Inflammatory mechanisms are not a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2002, 165:1206–1207; discussion 1207–1208
20. Flaherty KR, Thwaite EL, Kazerooni EA, Gross BH, Toews GB, Colby TV, Travis WD, Mumford JA, Murray S, Flint A, Lynch JP III, Martinez FJ: Radiological versus histological diagnosis in UIP and NSIP: survival implications. *Thorax* 2003, 58:143–148
21. Agostini C, Gurrieri C: Chemokine/cytokine cocktail in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* 2006, 3:357–363
22. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999, 103:779–788
23. Jakubzik C, Kunkel SL, Puri RK, Hogaboam CM: Therapeutic targeting of IL-4- and IL-13-responsive cells in pulmonary fibrosis. *Immunol Res* 2004, 30:339–350
24. Belperio JA, Dy M, Burdick MD, Xue YY, Li K, Elias JA, Keane MP: Interaction of IL-13 and C10 in the pathogenesis of bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2002, 27:419–427
25. Choi ES, Pierce EM, Jakubzik C, Carpenter KJ, Kunkel SL, Evanoff H, Martinez FJ, Flaherty KR, Moore BB, Toews GB, Colby TV, Kazerooni EA, Gross BH, Travis WD, Hogaboam CM: Focal interstitial CC chemokine receptor 7 (CCR7) expression in idiopathic interstitial pneumonia. *J Clin Pathol* 2006, 59:28–39
26. Kellermann SA, Hudak S, Oldham ER, Liu YJ, McEvoy LM: The CC chemokine receptor-7 ligands 6CKine and macrophage inflammatory protein-3 beta are potent chemoattractants for in vitro- and in vivo-derived dendritic cells. *J Immunol* 1999, 162:3859–3864
27. Jakubzik C, Choi ES, Joshi BH, Keane MP, Kunkel SL, Puri RK, Hogaboam CM: Therapeutic attenuation of pulmonary fibrosis via targeting of IL-4- and IL-13-responsive cells. *J Immunol* 2003, 171:2684–2693
28. Jakubzik C, Choi ES, Kunkel SL, Joshi BH, Puri RK, Hogaboam CM: Impact of interleukin-13 responsiveness on the synthetic and proliferative properties of Th1- and Th2-type pulmonary granuloma fibroblasts. *Am J Pathol* 2003, 162:1475–1486
29. Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, Phan SH: Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. *J Immunol* 1994, 153:4733–4741
30. Ryu JH, Colby TV, Hartman TE: Idiopathic pulmonary fibrosis: current concepts. *Mayo Clin Proc* 1998, 73:1085–1101
31. Jakubzik C, Wen H, Matsukawa A, Keller M, Kunkel SL, Hogaboam CM: Role of CCR4 ligands, CCL17 and CCL22, during *Schistosoma mansoni* egg-induced pulmonary granuloma formation in mice. *Am J Pathol* 2004, 165:1211–1221
32. Banas B, Wornle M, Berger T, Nelson PJ, Cohen CD, Kretzler M, Pfistering J, Mack M, Lipp M, Grone HJ, Schlondorff D: Roles of SLC/CCL21 and CCR7 in human kidney for mesangial proliferation, migration, apoptosis, and tissue homeostasis. *J Immunol* 2002, 168:4301–4307
33. Bonacchi A, Petrai I, Defranco RM, Lazzeri E, Annunziato F, Efsen E,

- Cosmi L, Romagnani P, Milani S, Failli P, Batignani G, Liotta F, Laffi G, Pinzani M, Gentilini P, Marra F: The chemokine CCL21 modulates lymphocyte recruitment and fibrosis in chronic hepatitis C. *Gastroenterology* 2003, 125:1060–1076
34. Jenh CH, Cox MA, Kaminski H, Zhang M, Byrnes H, Fine J, Lundell D, Chou CC, Narula SK, Zavodny PJ: Cutting edge: species specificity of the CC chemokine 6CKine signaling through the CXC chemokine receptor CXCR3: human 6CKine is not a ligand for the human or mouse CXCR3 receptors. *J Immunol* 1999, 162:3765–3769
35. Hammad H, Lambrecht BN, Pochard P, Gosset P, Marquillies P, Tonnel AB, Pestel J: Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *J Immunol* 2002, 169:1524–1534
36. Skovseth DK, Yamanaka T, Brandtzaeg P, Butcher EC, Haraldsen G: Vascular morphogenesis and differentiation after adoptive transfer of human endothelial cells to immunodeficient mice. *Am J Pathol* 2002, 160:1629–1637
37. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN: Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 2001, 166:7556–7562
38. Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH: Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004, 113:243–252
39. Kimura M, Tani K, Miyata J, Sato K, Hayashi A, Otsuka S, Urata T, Sone S: The significance of cathepsins, thrombin and aminopeptidase in diffuse interstitial lung diseases. *J Med Invest* 2005, 52:93–100
40. Wattiez R, Hermans C, Cruyt C, Bernard A, Falmagne P: Human bronchoalveolar lavage fluid protein two-dimensional database: study of interstitial lung diseases. *Electrophoresis* 2000, 21:2703–2712
41. Pardo A, Selman M: Matrix metalloproteases in aberrant fibrotic tissue remodeling. *Proc Am Thorac Soc* 2006, 3:383–388
42. Kuhn C, Mason RJ: Immunolocalization of SPARC, tenascin, and thrombospondin in pulmonary fibrosis. *Am J Pathol* 1995, 147:1759–1769
43. Adachi K, Yamauchi K, Bernaudin JF, Fouret P, Ferrans VJ, Crystal RG: Evaluation of fibronectin gene expression by in situ hybridization: differential expression of the fibronectin gene among populations of human alveolar macrophages. *Am J Pathol* 1988, 133:193–203
44. Mauch C: Matrix metalloproteinase-19: what role does this enzyme play in wound healing? *J Invest Dermatol* 2003, 121:xix–xx