New concepts of IL-10-induced lung fibrosis: fibrocyte recruitment and M2 activation in a CCL2/CCR2 axis

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Sun L, Louie MC, Vannella KM, Wilke CA, LeVine AM, Moore BB, Shanley TP. New concepts of IL-10-induced lung fibrosis: fibrocyte recruitment and M2 activation in a CCL2/CCR2 axis. Am J Physiol Lung Cell Mol Physiol 300: L341–L353, 2011. First published December 3, 2010; doi:10.1152/ajplung.00122.2010.—IL-10 is most commonly recognized as an anti-inflammatory cytokine possessing immunosuppressive effects necessary for regulated resolution of proinflammation. However, its role in the development of fibrosis during inflammatory resolution has not been clear. Few prior studies have linked IL-10 with the inhibition of fibrosis principally on the basis of regulating inflammation thought to be driving fibroproliferation. In contrast, in a model of long-term overexpression of IL-10, we observed marked induction of lung fibrosis in mice. The total cell number retrieved by bronchoalveolar lavage (BAL) increased 10-fold in the IL-10 overexpression (IL-10 OE) mice, with significant infiltration of T and B lymphocytes and collagen-producing cells. The presence of increased fibrocytes, isolated from collagenase-digested lungs, was identified by flow cytometry using dual staining of CD45 and collagen I. Quantitative PCR analysis on an array of chemokine/chemokine receptor genes showed that receptor CCR2 and its ligand, CCL2, were highly upregulated in IL-10 OE mice, suggesting that IL-10-induced fibrocyte recruitment was CCL2/CCR2 specific. Given the prior association of alternatively activated (M2) macrophages with development of fibrosis in other disease states, we also examined the effect of IL-10 OE on the M2 macrophage axis. We observed significantly increased numbers of M2 macrophages in both BAL and whole lung tissue from the IL-10 OE mice. Administration of rabbit anti-CCL2 antisera to IL-10 OE mice for three consecutive weeks significantly decreased fibrosis as evidenced by lung hydroxyproline content, compared with mice that received preimmune rabbit serum. These results indicate that overexpression of IL-10 induces fibrosis, in part, by fibrocyte recruitment and M2 macrophage activation, and likely in a CCL2/CCR2 axis.

inflammation; alveolar macrophage; chemokine

IL-10 is most well known as a canonical anti-inflammatory cytokine. This role has been delineated from several lines of investigation demonstrating its immunosuppressive effect and consequent regulation of proinflammation; this role appears to be a necessary component of resolution of inflammation. For example, we (37) previously showed that short-term, lung-specific overexpression of IL-10 attenuated LPS-induced acute inflammation. Interestingly, in these studies, longer term overexpression not only increased T-cell content in the lungs but also induced upregulation of Th2 response mediators in the lung, such as CCL2, CCL3, and CCL5 (37). Given these observations and the findings that IL-10 has been associated with the development of fibrosis in other organs (e.g., skin and kidney), we aimed to further elucidate the role of IL-10 on the development of lung fibrosis, which has remained controversial (1, 8, 20, 31).

Pulmonary fibrosis is characterized by the progressive deposition of extracellular matrix (ECM) components such as collagen and fibronectin. Cells responsible for the production of these matrix components include fibroblasts and myofibroblasts, which can be derived from three potential sources: the expansion of resident lung fibroblasts, the process of epithelial-mesenchymal transition, or the recruitment and differentiation of circulating mesenchymal precursors known as fibrocytes (19, 38). Recent studies (2, 3, 26, 27, 29) recognized that bone marrow-derived fibrocytes play an important role in tissue repair and fibrosis. Fibrocytes are circulating leukocytes that are characterized by their expression of CD45, CD34, CD13, and Arg1, and ST2 markers (10, 24, 25). This is important as IL-10 has been shown to induce fibrocyte recruitment in vivo (2, 3, 26, 27, 29). Structure of this cytokine drives the recruitment of fibrocytes include CCL2, CCL12 (murine homolog of human CCL2), CCL19, CCL21b, and CXCL12 (29, 33, 34). Corresponding receptors for these ligands, CCR2, CCR7, and CXCR4, are found on fibrocytes (27, 28, 33, 34). Results from receptor blockade studies or receptor gene knockout studies showed that with decreased fibrocyte recruitment, fibrotic responses were attenuated (7, 26, 28, 34).

Further evidence linking IL-10 to a potential role in fibrosis has been an increasing recognition that fibrosis has been associated with a strong Th2 cytokine milieu in models such as bleomycin- and helminth-induced lung fibrosis (16, 21, 35). These studies have suggested that in a Th2-dominant environment, macrophages are polarized to an alternatively activated (M2) phenotype, characterized by the upregulation of FIZZ1, Arg1, and ST2 markers (10, 24, 25). This is important as IL-10 is known to both inhibit classical activation and enhance alternative activation of macrophages (10, 17, 25); however, there are very few studies that have examined the effect of IL-10 in modulating the immune environment in a manner that strongly favors the development of fibrosis. Utilizing an in vivo model of lung-specific, IL-10 overexpression, we were able to test the hypothesis that IL-10 drives fibrocyte recruitment to the lung via chemokine/chemokine receptor expression targeting these cells and the development of alternatively activated macrophages to drive development of lung fibrosis. The results have important implications for putative roles of IL-10 during the course of resolution of inflammation in both acute and chronic inflammatory disease states.
MATERIALS AND METHODS

**Animals.** All in vivo studies were performed in compliance with and in accordance to the National Institutes of Health Guidelines using protocols approved by the University of Michigan’s Committee on Use and Care of Animals. The tetracycline-inducible, lung-specific human IL-10-overexpression (OE) transgenic FVB/n mice have been previously described (37). Chow containing tetracycline (0.0625%) was purchased as TestDiet from Purina (Richmond, IN). With this methodology, we achieve lung-specific, human IL-10 overexpression at about half-maximal levels at day 3 and maximal expression levels by day 5 following ad libitum access to TestDiet (37). Previous investigations have characterized all potential control mice (FVB/n wild-type and each single transgenic mouse line), none of which demonstrated tetracycline-inducible human IL-10 expression. Thus, for these experiments, single transgenic FVB/n mice possessing only the tetracycline-responsive, human IL-10 construct (tet-O-CMV-huIl-10), but not the CC10 rtTA transgene (designated as “control” mice) and bitransgenic (IL-10 OE) mice, were provided with ad libitum access to TestDiet. All data described in RESULTS (see Figs. 1–8), unless specifically stated, were obtained from 8- to 12-wk-old mice that were fed with tetracycline-containing chow for 1 mo.

**Bronchoalveolar lavage.** Mice were anesthetized with ketamine-HCl (150 mg/kg ip), and the trachea was exposed in a sterile manner. Bronchoalveolar lavage (BAL) was performed by instilling 1 ml normal saline, followed by gentle suction with an ~0.8-ml volume return. BAL cells were collected using repetitive (3 times) instillation and withdrawal of 1 ml PBS. Pooled BAL samples were centrifuged at 1,500 rpm for 10 min, and cell pellets were resuspended in RPMI +10% FCS and plated in 12-well plates. Alveolar macrophages were allowed to adhere at 37°C for 2 h, and nonadherent cells were removed. This procedure resulted in >95% purity of macrophages in culture.

**Assessment of gene expression by real-time PCR.** Total RNA was isolated from cultured BAL cells or whole lung tissue with the Trizol method (Invitrogen Life Technologies) according to the manufacturer’s protocol. Then, 1 μg of total RNA was reverse transcribed in a 20 μl volume. Messenger RNA expression was determined in 2 μl of cDNA by TaqMan real-time PCR using a Realplex detection system (Eppendorf). The primers and probes used for the TaqMan reaction were purchased from Applied Biosystems. Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 sec. Murine GAPDH (Applied Biosystems) was used as an internal control to quantify the total amount of cDNA used in the reaction. Cyber green real-time PCR was used to amplify collagen 1, collagen 2, fibronectin, ST2, CCL19, CCL21b, and CCR2. Primers for these genes were either synthesized as previously described (16, 28) or purchased from SABiosciences. Results are normalized to GAPDH expression and presented as fold increase in mRNA expression compared with the level detected in control mice.

**Western blots.** One million pooled BAL cells were washed with ice-cold PBS and lysed with 1× RIPA buffer containing 1/100 dilution of halot protease inhibitor cocktail (Thermo Scientific). Twenty-micrograms of protein from each group were analyzed for expression of fibronectin, collagen 1, FIZZ1, and Arg1 using methods that have been described previously (40). Blots were stripped and reprobed for GAPDH. Anti-mouse fibronectin (sc-6952) and anti-mouse Arg1 (sc-20150) polyclonal antibodies were purchased from Santa Cruz Biotechnology.

**ELISA.** Murine MCP-1 and IL-13 levels in BAL and whole lung homogenates were measured by standard ELISA technique.

**Assessment of lung pathology and lung collagen measurements.** Total lung collagen levels were determined by harvesting lungs from mice fed tetracycline chow for a month. Animals were euthanized and lungs perfused via the right ventricle using PBS until the pulmonary vessels were grossly clear. Lungs were inflated with 1 ml of 10% neutral buffered formalin and fixed overnight. After dehydration in 70% ethanol, the lungs were processed using standard procedures and embedded in paraffin. Sections were cut, mounted on slides, and stained with Masson’s trichrome. Under identical experimental conditions, total lung collagen levels were measured with the previously described hydroxyproline method (28).

**Immunohistochemical staining for FIZZ1 and F4/80.** Sections of paraffin-embedded lung lobes were deparaffinized and rehydrated. Epitope retrieval was performed by microwaving the sections in citrate buffer (pH 6.0) for 10 min. Sections were reacted with hydrogen peroxide block and then incubated with either rabbit anti-mouse FIZZ1 polyclonal antibody (AbCam; 1:200 dilution) overnight at 4°C or rat anti-mouse F4/80 monoclonal antibody (AbD serotec; 1:200 dilution) at room temperature for 30 min. The tissue sections were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (EnVision++; DAKO North America) at room temperature for 30 min and then stained with DAB (DAB Peroxidase System) for 5 min for F4/80 staining. For F4/80 staining, tissue sections were first incubated with biotinylated rabbit-anti-rat IgG (H+L) for 30 min (Vector Laboratories) and then incubated with DAKO streptavidin-labeled tertiary reagent for 30 min, followed by a 5-min staining with DAB. For both FIZZ1 and F4/80 staining, the sections were counterstained with hematoxylin for 1–3 s before dehydration, Xylene clearing, and the final mounting.

**Pulmonary leukocyte isolation and flow cytometry analysis of fibrocytes.** Animals were euthanized by approved protocol, and the lungs were perfused with PBS via the right heart until pulmonary vessels were grossly clear. Lungs were bluntly dissected free from the chest cavity and minced to a slurry in a suspension of digest solution containing collagenase (15 mg), DNase I (250 KU units), and complete media (RPMI-10% FCS). The suspension was incubated on a rocker for 30 min at 37°C. The cells were dispersed by repetitive suction through a 10-cc syringe and then centrifuged at 1,100 rpm for 10 min. After the supernatant was decanted, each pellet was briefly resuspended with 1 ml sterile ddH2O to lyse red blood cells and then recentrifuged. Cell pellets were resuspended in 5 ml of complete media and passed through a 70-μm cell strainer. Cells were then counted using a Beckman Coulter counter.

**Leukocytes were incubated for 15 min on ice with Fc block (BD Pharmingen) before surface staining with CD45-PerCP Cy5.5 (BD Pharmingen). Cells were then washed and fixed/permeabilized using the Cytofix/Cytoperm kit from BD Pharmingen and stained for collagen I (rabbit anti-mouse collagen 1; Rockland Immunochemicals) followed by a donkey anti-rabbit PE-coupled secondary (Jackson ImmunoResearch). Each sample was also stained with control rabbit IgG purchased from Jackson ImmunoResearch Laboratories. The percentage of fibrocytes was calculated by subtracting the percentage of cells stained with the irrelevant control IgG from the specific collagen 1 staining percentage.

**Flow cytometry analysis of BAL cells.** BAL cells (50,000 cells) in 100 μl flow assay buffer were first incubated with 0.2 μg of mouse Fc blocker (BD Pharmingen) for 15 min and then stained with CD19-PE, CD3-PE, and F4/80-Alexa 488 (Biolegend) for 30 min at 4°C. After being washed, the cells were resuspended in FACS buffer (1× PBS, 0.2% BSA, and 0.1% sodium azide) and analyzed on a flow cytometer (Accuri C6; Accuri Cytometers).

In the in vitro stimulation experiment, BAL cells were incubated with control or 20 ng/ml of recombinant IL-10 or IL-13 (R&D Systems) for 3 days before flow analysis. After Fc blocking, cells were either directly stained with fluorochrome-conjugated antibodies [CD206 (MMR)-Alexa 647 (Biolegend); CD11c-PE and CD11b-FITC (BD Pharmingen)] or were first incubated with Biotin-conjugated anti-mouse I-Ab for 30 min and then incubated with streptavidin-APC (Biolegend) for 20 min at 4°C. Each sample was also stained with isotype control antibody for flow analysis.

**Immunochemistry staining of collagen 1, FIZZ1, and Arg1.** BAL cells were combined and plated in six-well plates (2 million
cells/well). Cells were allowed to grow in complete medium for 14 days until the colonies of fibroblast-like cells reached confluency. The cells were then trypsinized and plated in eight-well chamber slides overnight. For intracellular collagen 1 staining, cells were fixed with 4% paraformaldehyde and permeabilized with ethanol. Next, the cells were washed and endogenous peroxidase blocked before incubation with rabbit anti-collagen 1 IgG (Rockland Immunochemicals) at a dilution of 1:200 for 30 min. Thereafter, the tissue sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (EnVision+; DAKO North America) at room temperature for 30 min and stained with DAB for 5 min before counterstaining with hematoxylin.

BAL cells isolated from control and IL-10 OE mice were plated in eight-well chamber slides overnight. The same procedure as collagen 1 staining was used for FIZZ1 and Arg1 immunocytochemistry analysis.

**CCL2 blocking experiment.** Antiserum containing polyclonal antibodies directed against mouse CCL2 was kindly provided by Dr. Steven Kunkle’s laboratory at the University of Michigan. The specificity of the anti-CCL2 antiserum has been demonstrated to lack cross-reactivity with all other chemokines and cytokines (6). Three days after tetracycline chow feeding, both control mice and IL-10 OE mice were injected intraperitoneally with either 0.5 ml of preimmune rabbit serum or an equivalent amount of anti-CCL2 immune serum. Mice were given intraperitoneal injection every 1 wk after with a total injection of three times. One month posttetracycline chow, lungs were harvested and subjected to hydroxyproline assay for measurement of total lung collagen levels.

**Statistical analysis.** All statistics were performed using Graphpad Prism 4 (San Diego, CA). Values are expressed as means ± SE. Significance was assigned for \( P < 0.05 \). Data sets were analyzed...
using Student’s t-test or one-way ANOVA, with individual group means being compared with the Student-Newman-Keuls posttest.

RESULTS

IL-10 overexpression induces fibrosis in the lung. Previously, we (37, 39) demonstrated that short-term overexpression of IL-10 in the lung attenuated LPS-induced acute inflammation and decreased bacterial clearance. To investigate the long-term effects of IL-10 overexpression, we fed transgenic mice tetracycline-spiked chow for 1 and 2 mo to induce IL-10 (designated IL-10 OE) and examined the lung for pathological changes. Compared with single transgenic mice also provided ad libitum access to tetracycline-spiked chow treated under the same experimental conditions (designated TG⁺), IL-10 OE mice exhibited substantial cellular accumulation around small and large airways (Fig. 1A). Furthermore, trichrome staining showed strong collagen deposition in the peribronchial areas and around pulmonary arteries, indicating that long-term IL-10 overexpression induced fibrosis (Fig. 1A, bottom) that was not observed in TG⁻ mice. Furthermore, to examine whether IL-10 OE-induced fibrosis is reversible, overexpression mice were switched to regular chow after 1 mo of tetracycline treatment and maintained for another month. At 1 mo, removal of tetracycline did not reverse the fibrotic phenotype in the IL-10 OE mice (Fig. 1A, right). At 2 mo of tetracycline treatment, IL-10 OE-induced fibrosis remained peribronchial with no obvious extension towards alveolar air spaces (Fig. 1A, middle; IL-10 OE doxy-2mon ×400). To more specifically quantify collagen production, hydroxyproline assay was performed and showed a nearly twofold increase in collagen production from the IL-10 OE lungs (Fig. 1B). Again, the IL-10 OE-induced fibrotic effects appeared irreversible, as similar hydroxyproline levels were observed in the tetracycline chow-removal group compared with mice that were continuously fed with tetracycline for 2 mo (Fig. 1B).

Fig. 2. IL-10 overexpression induces lymphocytic infiltration into the alveolar space. BAL cells were harvested by repeated lavage as described in MATERIALS AND METHODS. A: lymphocytic cell population in the IL-10 OE mice was identified on the FSC/SSC plot. B: frequency of macrophages, T cells, and B cells from the bronchoalveolar lavage (BAL) fluids were determined by surface markers F4/80, CD3 and CD19 by flow cytometry. C: total BAL cell numbers retrieved from control mice and IL-10 OE mice. Results are ± SE of 3 animals and represent 3 independent experiments of a total number of 9–10 individual animals per group.
**IL-10 overexpression changed the cellular composition of BAL.** Because the histology of IL-10 OE lung displayed significantly increased cellular infiltration, we aimed to enumerate and characterize the cells migrating into the alveolar spaces as retrieved BAL. In TG− mice, the majority of the BAL cells were macrophages (macrophage population in the FSC/SSC plot; Fig. 2A), represented as F4/80-positive cells determined by flow cytometry (Fig. 2B). However, in BAL from the IL-10 OE mice, a unique lymphocyte population appeared (Fig. 2A), which decreased the macrophage composition to 50% as opposed to 95% in the control mice BAL (Fig. 2B). This lymphocyte population included both CD3-positive T cells and CD19-positive B cells. Overall, the total cell numbers were increased by 10-fold (0.18 ± 0.041 × 10^6/IL-10 OE mice; Fig. 2C).

IL-10 overexpression induced collagen production from BAL cells. Having observed that IL-10 overexpression changed the cellular composition of BAL cells, we next wanted to determine whether BAL cells from the IL-10 OE mice were able to produce extracellular matrix proteins, such as collagen 1, collagen 3, and fibronectin. To do this, we performed in vitro culturing of retrieved BAL cells from IL-10 OE (vs. TG− mice) as described in MATERIALS AND METHODS. After allowing cellular adherence for 2 h and removing nonadherent cells, adherent cells were collected for RNA isolation and quantitative (Q)-PCR analysis for collagen 1, collagen 3, and fibronectin. As shown in Fig. 3A, all three matrix genes were highly upregulated in adherent cells isolated from IL-10 OE mice compared with TG− mice. Protein expression of collagen 1 and fibronectin was examined by Western blot analysis. As shown in Fig. 3B, both collagen 1 and fibronectin were detected in cells isolated by BAL from IL-10 OE mice, whereas no signal was detected in cells isolated from the TG− mice. These data suggested that IL-10 overexpression recruited extracellular matrix-producing cells to the BAL; however, flow cytometry analysis failed to detect specific collagen-producing cells from the BAL, which we surmised might be due to the low percentages of these cells or the weak expression of collagen 1 in these cells recovered by BAL. Therefore, we used an alternative strategy of in vitro culturing the BAL cells for 14 days to allow the cells to proliferate and subsequently performed immunocytochemistry analysis on the cultured cells (Fig. 3C). With the use of this strategy, a significant number of collagen-producing cells from IL-10 OE mice were identified by staining with anti-collagen 1 antibody. As shown in Fig. 3C, these collagen 1 positive cells also exhibited a classic spindle shape, consistent with a fibrocyte phenotype (38). Intriguingly, after this 14-day period of in vitro culture, while ~90% of the macrophages isolated in BALs from IL-10 OE mice remained alive, no macrophages retrieved from BALs of TG− mice survived (data not shown). This observation suggested an alternative activation state of the macrophages isolated from IL-10 OE mice.

**IL-10 overexpression induced alternative activation of macrophages.** IL-10 is a known regulator of M2 activation (25). To demonstrate whether this was occurring in this in vivo model, we performed Q-PCR analysis on the adherent BAL...
Fig. 4. IL-10 overexpression induces alternative activation of macrophages. Both control and IL-10 OE mice were fed with tetracycline-containing chow for 1 mo. BAL cells harvested from control and IL-10 OE mice were allowed to adhere for 2 h at 37°C and then, after removal of suspended cells, were subjected to total RNA preparation and Q-PCR analysis for FIZZ1, Arg1 and ST2 gene expression (A) or Western blot analysis for Arg1 and FIZZ1 protein expression (B), and immunocytochemistry analysis with anti-FIZZ1 antibody (C, top) and anti-Arg1 antibody (C, bottom) for intracellular staining of FIZZ1 and Arg1. Magnification: ×400. D: lung immunohistochemical staining of F4/80 and FIZZ1. Paraffin-embedded lung sections were subjected to immunohistochemistry staining with anti-F4/80 and anti-FIZZ1 antibodies to distinguish lung macrophages and to identify alternative activated macrophages. Stronger signals and accumulation of F4/80-positive cells were found around the bronchial epithelial areas in IL-10 OE mice. While control mice showed negative staining of FIZZ1, FIZZ1 was highly expressed on bronchial epithelial cells and in the infiltrated macrophages from the IL-10 OE mice. Magnification: ×200 and ×100.

A: M2 marker genes

- TG-
- IL-10 OE

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<th>Fold Increase</th>
<th>TG-</th>
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<tr>
<td>FIZZ1</td>
<td>30</td>
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B: Western blot analysis

- 40KDa: Arg1
- 20KDa: FIZZ1
- 30KDa: GAPDH

C: Immunocytochemistry

- Anti-FIZZ1 x 400
- Anti-Arg1 x 400

- TG-
- IL-10 OE

- IL-10 OE
- Anti-F4/80
- Anti-FIZZ1
cells for classic M2 marker genes, Arg1, FIZZ1, and ST2. All these M2 markers were highly upregulated in the cells isolated from IL-10 OE mice by BAL (Fig. 4A). This quantitative RNA data were further confirmed by western blot (Fig. 4B) and immunocytochemistry staining (Fig. 4C) with anti-FIZZ1 antibody and anti-Arg1 antibody. Immunocytochemistry identified the presence of FIZZ1-positive (Fig. 4C, top) and Arg1-positive (Fig. 4C, bottom) macrophages in situ in the IL-10 OE mice but not TG− mice. Thus these positively stained macrophages are consistent with an M2 macrophage phenotype. In contrast to classically activated macrophages, some of the alternatively activated macrophages exhibited enlarged cell size (Fig. 4C). To further characterize the activated macrophages in lung tissue, we did parallel staining with antibodies against the macrophage marker F4/80 (Fig. 4D, left) and the M2 marker FIZZ1 (Fig. 4D, right). While in TG− mice there were very few interstitial and alveolar macrophages present (represented as F4/80-positive cells), profound macrophage infiltration was found in the bronchial airway areas of IL-10 OE mice (Fig. 4D, left middle and bottom). Parallel staining of the same paraffin-embedded sections using anti-FIZZ1 antibody showed strong FIZZ1 expression in the bronchial epithelial cells in the IL-10 OE mice but not the TG− mice (Fig. 4D, right middle and bottom), notably in sections with clear development of fibrosis (by trichrome staining; data not shown). This is consistent with the reports that FIZZ1 is expressed by epithelial cells in fibrotic lungs (21). Moreover, a significant number of infiltrating macrophages also showed positive staining of FIZZ1, indicating that IL-10 overexpression not only appeared to recruit macrophages into the lung but also induced alternatively activated (M2) macrophages. IL-10 overexpression induced fibrocyte recruitment into the lung. It has recently been demonstrated that recruitment of fibrocytes from the circulation plays an important role in the
development of fibrosis. To examine whether infiltrating cells in the IL-10 OE mice included fibrocytes that might not be retrievable by BAL, we performed flow cytometry analysis of leukocytes isolated from digested whole lungs. The flow plots shown in Fig. 5, A and B, illustrate our strategy of fibrocyte identification. First, dead cells and debris were excluded by setting a gate (P1) on the FSC/SSC plot. Second, all stromal cells, including fibroblasts and epithelial cells were excluded by setting a gate on the leukocyte marker CD45 (P2). Finally, the percentage of fibrocytes was calculated by subtracting the percentage of cells stained with the irrelevant control IgG from those showing specific collagen 1 staining. In this manner, although we did not detect a change in the percentage of the fibrocytes between the control mice and the IL-10 OE mice (~2.5% each; Fig. 5, A and B), since the total number of leukocytes in the IL-10 OE mice was increased by twofold, the total number of fibrocytes in the IL-10 OE mice was doubled compared with TG− mice (0.21 ± 0.047 × 10^6/control mice compared with 0.45 ± 0.054 × 10^6/IL-10 OE mice; Fig. 5C).

**IL-10 OE-induced fibrocyte recruitment is CCL2/CCR2 dependent.** A number of chemokines and their receptors have been implicated in the recruitment of fibrocytes into the lung during the development of fibrosis (28, 38, 41). To identify the specific chemokine that drives IL-10 recruitment of fibrocytes, we performed Q-PCR analysis on the adherent BAL cells for chemokine receptor gene expression and on the whole lungs for chemokine gene expression. Our data show that three receptors, CCR2, CCR7, and CXCR4, were significantly upregulated on the cells retrieved by BAL from the IL-10 OE mice compared with TG− mice (Fig. 6A). In RNA isolated from whole lung tissue, only the ligand for CCR2 (CCL2) was highly upregulated in IL-10 OE mice.

**Fig. 5.** IL-10 overexpression induces recruitment of fibrocytes into the lung. A, B: identification of lung fibrocytes. Whole lung leukocytes were isolated by collagenase digestion method as described in MATERIALS AND METHODS. After surface staining with CD45-PerCP Cy5.5, cells were fixed and permeabilized with paraformaldehyde and 0.1% saponin, followed by intracellular staining with either control rabbit IgG (A) or anti-collagen 1 rabbit IgG (B). Finally, cells were stained with donkey anti-rabbit PE-coupled secondary and subjected to flow cytometry analysis. Percentage of fibrocytes was calculated by subtracting the percentage of cells stained with the irrelevant control IgG from the specific collagen 1 staining percentage. C: total lung fibrocyte numbers. Lung fibrocyte numbers = total lung leukocyte numbers × fibrocyte percentage. Data represent n = 4 per group and were repeated 3 times.
compared with lungs from TG− mice (~35-fold increase), whereas the ligands for CCR7 (CCL19 and CCL21b) and CXCR4 (CXCL12) were not different between the two mice groups. The amount of CCL2 released to the BAL fluid of IL-10 OE mice was also highly increased, which was determined by ELISA (Fig. 6C). These results suggested that IL-10 overexpression-induced fibrocyte recruitment is likely mediated by CCL2/CCR2 interactions.

In vitro M2 activation by IL-10 in cultured BAL macrophages. Previously, Lee et al. (20) reported that IL-13 was upregulated in a continuous lung IL-10 overexpression model. Consistent with their report, we also observed highly up-regulated IL-13 expression from both BAL cells and whole lung tissues retrieved from the inducible IL-10 OE mice (Fig. 7, A and B). To examine whether alternative activation of macrophages present in the IL-10 OE mice was induced by IL-10 directly or was due to the high levels of IL-13 present in these mice, we isolated BAL macrophages from wild-type mice and treated them with 20 ng/ml of recombinant mouse IL-10 for 2 and 4 consecutive days (Fig. 7C, left). As a comparison, BAL macrophages were also treated with mouse IL-13 and examined for M2 marker gene expression (Fig. 7C, right). Q-PCR analysis showed that exposure to IL-10 alone induced Arg1 and ST2 but not Ym1 expression in the cultured cells (Fig. 7C, left). Under this experimental condition, FIZZ1 was too low to be detectable. IL-13, on the other hand, strongly stimulated Arg1 and Ym1 expression and moderately upregulated ST2 expression (Fig. 7C, right). Immunocytochemistry was performed to examine Arg1 expression in BAL macrophages in situ. As shown in Fig. 7D, a significant amount of Arg1-positive cells was detected in both IL-10- and IL-13-treated BAL cells, whereas control PBS-treated cells showed negative staining. We also performed flow analysis and compared expression of several surface markers between IL-10 and IL-13 treatment. In this experiment, cells were treated with IL-10 or IL-13 for 3 days before the flow assay. As shown in Fig. 7E, both cytokines upregulated mannose receptor (MMR) expression; however, only IL-13 upregulated MHC II, CD11c, and CD11b expression, whereas IL-10 did not. Therefore, M2 macrophages activated by IL-10 and IL-13 share some common features but also express distinct intracellular and cell surface profiles. Taken together, our data indicated that because IL-10 alone can induce M2 activation, two separate pathways may be involved in the induction of M2 macrophages in the IL-10 OE mice. While one pathway is mediated via IL-13 axis, the other is likely mediated in an IL-10-regulated but IL-13-independent manner.

Attenuation of IL-10 OE-induced fibrosis by anti-CCL2 blocking. In Fig. 6, we showed that CCL2/CCR2 was specifically and highly upregulated in the lungs of IL-10 OE mice. We therefore surmised that increased fibrocyte recruitment and M2 recruitment or activation might be regulated by the CCL2/CCR2 axis, thereby leading to the development of fibrosis. To ascertain this, we adapted an established CCL2 immunoneutralization experiment (6) to examine the effects of anti-CCL2 blocking on the fibrotic responses induced by IL-10 OE. In this experiment, during the 1 mo of feeding with tetracycline-containing chow, anti-CCL2 antiserum (vs. preimmune rabbit serum) was administered for three consecutive weeks. As shown in Fig. 8, compared with mice that received preimmune serum, administration of anti-CCL2 antiserum to IL-10 OE mice significantly decreased fibrosis, as evidenced by lung hydroxyproline content. In control mice, hydroxyproline levels remained the same between anti-CCL2 serum and normal serum-treated group (Fig. 8). This experiment established an associative link between IL-10 and the CCL2/CCR2 axis in playing a key role in the development of lung fibrosis in our model. Whether the cause of this attenuated fibrotic response is related to blocking of fibrocyte recruitment is the target of ongoing studies.
Although initially identified and primarily characterized as an anti-inflammatory cytokine, ongoing studies have shown IL-10 to possess several immune-modulating properties beyond deactivation of macrophage and dendritic cell function. For example, IL-10 was recently discovered to induce CXCL13 production from antigen-presenting cells to increase the recruitment of B cells and promote lymphoid...
tissue neogenesis (32). IL-10 was also found to upregulate prototypic long pentraxin 3 (PTX3) expression and thereby modulate ECM remodeling at sites of inflammation (23). In other ECM reconstruction-related diseases, such as COPD (18) and hypertrophic scar (HTS) and keloids (4), significantly increased IL-10 expression was present and these increased levels influenced subsequent wound healing. Despite these observations, there are few data characterizing the manner by which IL-10 might influence wound healing and the development of fibrosis, which is fundamentally important to understand given its critical role in resolving inflammation.

Previously, we (37) reported that overexpression of IL-10 in the lung induced cellular infiltration and the upregulation of several chemokines, but the biological and pathological effects were not examined. In the present study, we demonstrated that prolonged overexpression of IL-10 was sufficient to induce lung fibrosis. Given this observation, we sought to characterize the cellular source(s) of collagen-producing cells in this model. Because adherent cells isolated from IL-10 OE mice by BAL showed significant upregulation of collagen 1, collagen 3, and fibronectin compared with TG mice, we surmised that fibrocytes were being recruited into the alveolar space during the development of fibrosis. By subjecting isolated and adherent cells to in vitro culture for 14 days, we further confirmed the presence as well as the proliferation of spindle-shaped fibrocytes in the IL-10 OE mice (see Fig. 3), which were not present in normal control mice. On the basis of the total number of cells isolated from whole lung collagenase digests, we also found a twofold increase in the total number of fibrocytes, which are potent collagen-producing cells capable of differentiating into fibroblasts and myofibroblasts under certain conditions associated with the development of fibrosis (38). Therefore, we hypothesized that recruitment of fibrocytes into the lung could be an important mechanism by which IL-10 OE-induced fibrosis.

To identify the chemokines responsible for the IL-10-induced fibrocyte recruitment, we performed Q-PCR analysis on RNA isolated from whole lungs and found that only CCL2 was highly upregulated, whereas the other chemokines CCL19, CCL21b, and CXCL12 were unchanged. Correspondingly, we found a significant upregulation of the CCL2 receptor CCR2 in the adherent cells isolated by BAL from the IL-10 OE mice. Together, these data suggested that in our model CCL2 is the key mediator for the IL-10-induced fibrocyte recruitment to contribute to the development of fibrosis. By blocking CCL2 in vivo with anti-CCL2 antiserum, we found significantly decreased collagen levels in the IL-10 OE lungs compared with mice injected with normal serum (Fig. 8). Our experiment, together with other studies reporting that mice deficient in CCR2 (CCR2−/−) were protected from the development of FITC- or bleomycin-induced pulmonary fibrosis (9, 28, 30), demonstrates that the CCL2/CCR2 axis plays a key role in mediating the development of fibrosis in the IL-10 OE mice.

Relevant to the link between CCL2 expression and IL-10 overexpression is the known association of CCL2 with Th2 polarization. The role of CCL2 has been described in Th2 cytokine predominant diseases such as the immune response to Leishmania major (11), in eosinophil-rich interstitial lung granulomas induced by antigens of S. mansoni eggs (42), and in asthmatic lungs (12). CCL2 was also identified as an important profibrotic mediator (5, 22, 30). A direct link between profibrotic CCL2 activity and the production of Th2 cytokines such as IL-13 and IL-4 has been investigated. For example, in CCR2−/− mice or in mice injected with CCL2 blocking antibody, decreased IL-13/IL-4 production was observed (11, 43). On the other hand, both IL-13 and IL-4 were found to be potent inducers of CCL2. IL-13 was reported to upregulate CCL2 expression in human bronchial epithelial cells (14); similarly, in mouse macrophages, IL-4 induced CCL2 expression (24). In our study, we detected an ~35-fold increase in CCL2 expression from whole lung samples, while in the adherent BAL cells, no upregulation was detected (data not shown), suggesting that structural cells (e.g., epithelial cells) are likely to be the predominate source of CCL2. Of note, in our study, apoptosis assays performed on both whole lung sections and isolated epithelial cells did not detect increased apoptotic events with IL-10 OE (data not shown), excluding the likelihood that this fibrotic response was related to IL-10 OE-induced damage to epithelial cells.

In addition to fibrocyte recruitment, other cell types including T cells, B cells and macrophages also exhibited significant lung infiltration in the IL-10 OE mice. Importantly, IL-10 OE appeared to affect macrophage activation, as the infiltrating macrophages displayed an M2 phenotype. Furthermore, the CCL2/CCR2 pathway has been implicated in macrophage recruitment in diseases where M2 macrophages were critical mediators, such as obesity (15) and notably, tissue fibrosis (13). Thus we speculate that in addition to fibrocyte recruitment, CCL2/CCR2 is also the important mediator for this IL-10 OE-induced macrophage recruitment as well as M2 activation. It is important to note that in our study, we also detected significant upregulation of IL-13 from both the adherent BAL cells and mice whole lungs (Fig. 7, A and B). Both IL-13/IL-4 are known to be activators of M2 activation (24). Therefore, it is possible that the IL-10-induced fibrosis response is mediated by the IL-13/IL-4 pathway. However, in vitro exposure of BAL macrophages to recombinant IL-10 alone induced M2 (Fig. 7, C and D). Moreover, examination of surface marker expression showed that IL-10- and IL-13-induced macrophages have distinct expression profiles (Fig. 7E). Thus IL-10 can directly induce M2 activation and probably via different mechanisms from IL-13/IL-4 regulation. Un-
like IL-13/IL-4, very few studies have examined this potential role of IL-10 regulation (17, 36). To further define the roles of these Th2 cytokines with alternative activation of macrophages, IL-10 neutralizations studies as the next direction should aid in clarifying the regulation of the IL-10-induced fibrosis response from that mediated by IL-13/IL-4.

In conclusion, our results provide new mechanistic insight into the pathogenesis of fibrosis by demonstrating IL-10-induced fibrocyte recruitment is likely mediated by the CCL2-CCR2 axis. Additionally, we identified the involvement of M2 activation as part of the IL-10-induced fibrictic effect. These findings have important implications for the role of IL-10 in contributing to the pathophysiologic development of fibrosis in the settings where it is highly expressed to counter and resolve proinflammation, such as acute lung injury.

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