Regulatory T Cells Reduce Acute Lung Injury Fibroproliferation by Decreasing Fibrocyte Recruitment

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Acute lung injury (ALI) causes significant morbidity and mortality. Fibroproliferation in ALI results in worse outcomes, but the mechanisms governing fibroproliferation remain poorly understood. Regulatory T cells (Tregs) are important in lung injury resolution. Their role in fibroproliferation is unknown. We sought to identify the role of Tregs in ALI fibroproliferation, using a murine model of lung injury. Wild-type (WT) and lymphocyte-deficient $Rag\text{-}1^{-/-}$ mice received intratracheal LPS. Fibroproliferation was characterized by histology and the measurement of lung collagen. Lung fibrocytes were measured by flow cytometry. To dissect the role of Tregs in fibroproliferation, $Rag\text{-}1^{-/-}$ mice received $CD4^{+}CD25^{+}$ (Tregs) or $CD4^{+}CD25^{-}$ T cells (non-Tregs) at the time of LPS injury. To define the role of the chemokine (C-X-C motif) ligand 12 (CXCL12)–CXCR4 pathway in ALI fibroproliferation, $Rag\text{-}1^{-/-}$ mice were treated with the CXCR4 antagonist AMD3100 to block fibrocyte recruitment. WT and $Rag\text{-}1^{-/-}$ mice demonstrated significant collagen deposition on Day 3 after LPS. WT mice exhibited the clearance of collagen, but $Rag\text{-}1^{-/-}$ mice developed persistent fibrosis. This fibrosis was mediated by the sustained epithelial expression of CXCL12 (or stromal cell-derived factor 1 [SDF-1]) that led to increased fibrocyte recruitment. The adoptive transfer of Tregs resolved fibroproliferation by decreasing CXCL12 expression and subsequent fibrocyte recruitment. Blockade of the CXCL12–CXCR4 axis with AMD3100 also decreased lung fibrocytes and fibroproliferation. These results indicate a central role for Tregs in the resolution of ALI fibroproliferation by reducing fibrocyte recruitment along the CXCL12–CXCR4 axis. A dissection of the role of Tregs in ALI fibroproliferation may inform the design of new therapeutic tools for patients with ALI.

Keywords: acute lung injury; fibroproliferative ARDS; fibrocytes; regulatory T cells; lung injury resolution

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) affect 190,000 individuals in the United States each year, accounting for 75,000 deaths (1). The only treatment that improves outcomes involves a lung-protective strategy in

CLINICAL RELEVANCE

Fibroproliferation in the context of acute lung injury (ALI) leads to worse outcomes, but the factors that underlie the fibroproliferative phase of ALI remain unknown. We have shown that regulatory T cells reduce fibroproliferation after LPS-induced ALI by reducing fibrocyte recruitment to the lung along the CXCL12–CXCR4 axis. This may lead to the development of novel diagnostic and therapeutic strategies for patients with ALI.

patients on mechanical ventilation (2). Mortality from ALI/ARDS remains as high as 44% (3).

ALI/ARDS is divided into an exudative phase marked by edema fluid, hyaline membrane formation, and neutrophilic infiltration, followed in some patients by a fibroproliferative phase (4). Fibroproliferation is part of the normal repair response, and is characterized by the intra-alveolar accumulation of fibroblasts and collagen deposition. If this process is ineffective or continues unabated, patients may develop fibrosis (5). Longer durations of ARDS correspond to increased lung collagen and fibrosis, and portend worse outcomes (6). Fibroproliferative changes on biopsy and computed tomography predict mortality (7, 8). The determinants of prolonged fibroproliferation and factors that govern its resolution remain poorly understood.

The fibroblast is a key cell in fibroproliferation (9). The source of activated fibroblasts (myofibroblasts) in ALI remains unclear. Three cell populations likely contribute: (1) resident lung fibroblasts (10), (2) epithelial cells that transform into myofibroblasts via epithelial-to-mesenchymal transition (11), and (3) bone marrow progenitors called fibrocytes. Fibrocytes are identified by their expression of CD45, collagen-1 (Col-1), and CXCR4 (12–14). In a model of bleomycin fibrosis, fibrocytes accounted for 20% of myofibroblasts (10, 15). Fibrocytes are contained in the bronchoalveolar lavage (BAL) and blood of patients with idiopathic pulmonary fibrosis (IPF), and may predict exacerbations (14, 16). Recently, fibrocytes were found in the BAL of patients with ARDS, and were associated with increased mortality (17, 18). The mechanisms governing fibrocyte recruitment and activation in ALI remain unknown.

Persistent inflammation is a hallmark of fibroproliferative ARDS (4). Our group has highlighted a central role for regulatory T cells (Tregs, i.e., $CD4^{+}CD25^{+}Foxp3^{+}$ T cells) in the resolution of lung inflammation (19). The role of Tregs in the fibroproliferative response to ALI has not been described, to the best of our knowledge.

Using a well-established model of lung injury involving intratracheal LPS (20), we show that lymphocyte-deficient $Rag\text{-}1^{-/-}$ mice demonstrate persistent fibroproliferation, characterized by sustained fibrocyte recruitment to the lung. The adoptive transfer of Tregs, but not other $CD4^{+}$ T cells, into $Rag\text{-}1^{-/-}$ mice reduces

Figure 1. Adoptive transfer of T regulatory cells (Tregs; $CD4^+CD25^+$ cells) but not $CD4^+CD25^-$ cells into *Rag-$1^{-/-}$* mice restored normal resolution of lung injury. (*A*) Survival curves after intratracheal LPS for all groups (*$P = 0.021$ by survival log-rank, and #$P = 0.024$ by χ^2 test, $n \geqslant$ 14 in all groups). (*B*) Weight loss presented as percentage of baseline weight after intratracheal LPS for all groups (*$P < 0.001$, and #$P < 0.001$, $n \geqslant 14$, all groups). (*C*) Bronchoalveolar lavage (BAL) cell counts on Day 3 and Day 7 after intratracheal LPS or intratracheal water for all groups (*$P = 0.012$ and #$P < 0.05$, $n > 4$, all groups). (*D*) BAL protein measured by the method of Lowry on Day 3 and Day 7 after intratracheal LPS or intratracheal water for all groups (*$P = 0.001$ and #$P = 0.006$, $n \geqslant 4$ all groups). AT, adoptive transfer; WT, wild-type.

fibrocyte number and resolves fibroproliferation. This fibrocyte reduction is caused by a Treg-mediated decrease in lung CXCL12, a chemokine that induces fibrocyte recruitment. Epithelial cells are an important source of CXCL12, suggesting that Tregs reduce CXCL12 concentrations by interacting with the epithelium. The blockade of CXCR4, the receptor for CXCL12, decreases fibrocyte numbers and fibroproliferation in *Rag-$1^{-/-}$* mice, providing further evidence for the importance of the CXCL12–CXCR4 axis in ALI fibroproliferation. Our results indicate that Tregs play a critical role in the fibroproliferative response to ALI, and that targeting the CXCL12–CXCR4 pathway may provide new insights into the treatment of fibroproliferative ARDS.

Some of these results were previously reported in the form of an abstract (21).

MATERIALS AND METHODS

Mice

C57BL/6 wild-type (WT) and *Rag-$1^{-/-}$* mice (aged 6–8 wk) were purchased from Jackson Laboratories (Bar Harbor, ME). Green-fluorescent protein (GFP)-labeled, Foxp3$^+$ mice were the gift of Alexander Rudensky (Sloan-Kettering Institute, New York, NY). Protocols were approved by the Johns Hopkins Animal Care and Use Committee (Baltimore, MD).

Preparation of Mice

Mice were anesthetized with intraperitoneal ketamine and acetylpromazine (150 and 13.5 mg/kg, respectively) before tracheal exposure. *Escherichia coli* LPS (3.75 µg/g, O55:B5 L2880; Sigma-Aldrich, St. Louis, MO) or sterile water was instilled intratracheally via 20-gauge catheter. Animals were killed by vena cava exsanguination (19).

CXCR4 Blockade

Rag-$1^{-/-}$ mice underwent daily intraperitoneal injections of AMD3100 (200 µg/250 µl PBS; Sigma-Aldrich) or PBS (250 µl).

Isolation and Adoptive Transfer of Tregs

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from GFP-Foxp3$^+$ spleens using magnetic beads (Miltenyi, Cambridge, MA) (19). Single-cell suspensions (1×10^6 cells in 100 µl PBS) were transferred within 60 minutes after LPS by retro-orbital injection. Treg purity was measured at 85–90% by flow cytometry.

Analysis of BAL Fluid

Two aliquots of 0.7 ml PBS were instilled into the right lung. Cell counts were determined by hemocytometer. Protein was measured using the method of Lowry (19).

Lung Histology and Immunohistochemistry

Left lungs were inflated to 25 cm H_2O with 1% low-melting agarose (Invitrogen, Grand Island, NY) (19) for evaluation by Masson's trichrome. Sections were prepared for immunohistochemistry with anti–SDF-1 AB (1/50, clone 79018; R&D Systems, Minneapolis, MN), followed by a mouse-on-mouse immunodetection technique (Vector Laboratories, Burlingame, CA), peroxidase-conjugated secondary antibody, and development with 3,3-diaminobenzidine.

Lung and BAL Collagen Content

Left lungs were frozen in liquid nitrogen. Lungs were homogenized in 1 ml of Complete Lysis Buffer (Roche, Indianapolis, IN), using an Ultra-Turrax tissue homogenizer (Janke and Kunkel, Wilmington, NC). Collagen was measured by Sircol Assay (BioColor, Carrickfergus, UK), according to the manufacturer's instructions.

Lung CXCL12 Concentrations

CXCL12 concentrations were measured in lung homogenates, using an ELISA duoset (R&D Systems).

Preparation of Lung Homogenates for Flow Cytometry

Right lungs were excised, minced, and incubated at 37°C in RPMI containing 2.4 mg/ml collagenase I and 20 µg/ml DNase (Invitrogen), and then mashed through a 70-µm nylon strainer (BD Biosciences, San Jose, CA). For epithelial cell isolation, lungs were instilled with 1.2 ml Dispase (BD Biosciences), with 3 U/ml elastase (Worthington, Lakewood, NJ) at removal. Red blood cells were removed using ACK lysis buffer (Invitrogen), and cells were resuspended in FACS buffer.

Flow Cytometry

Cells were prepared for FACS analysis (19). Surface stains included Alexa-700 conjugated anti-CD4 (Ebioscience, San Diego, CA), allophycocyanin-conjugated (APC) anti-CXCR4 (BD Biosciences), APC-Cy7–conjugated anti-CD326, and biotinylated anti-CD45 and CD31 with V450-conjugated streptavidin secondary (Biolegend, San Diego, CA). Intracellular stains included biotinylated rabbit anti–Col-I
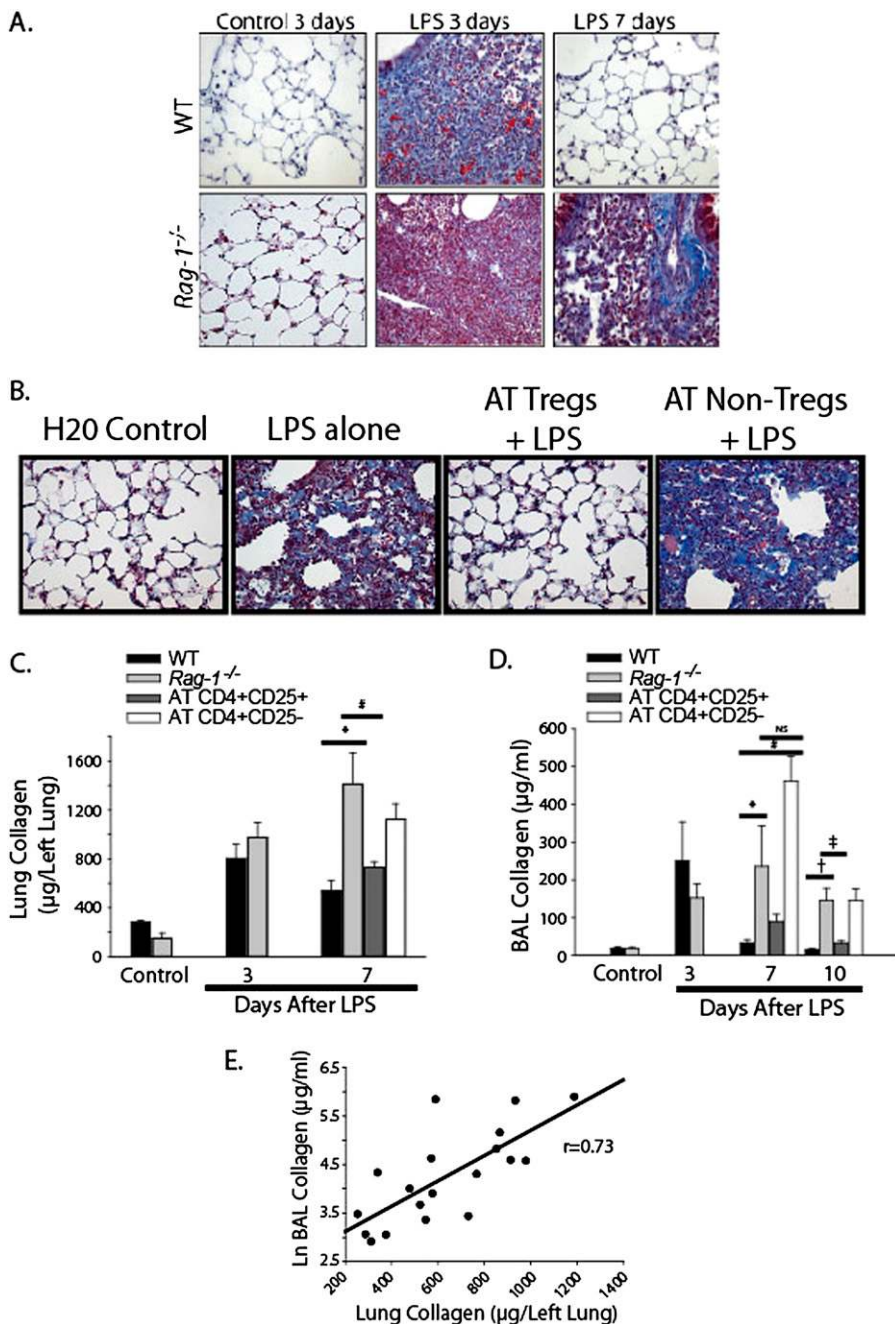
Figure 2. Adoptive transfer of Tregs reduced fibroproliferation in *Rag-1*$^{-/-}$ mice after LPS injury. (*A*) Lung sections were stained with Masson's trichrome to highlight collagen deposition on Day 3 and Day 7 after intratracheal LPS or intratracheal water in WT and *Rag-1*$^{-/-}$ mice (n ⩾ 3 in each group; images are of representative examples, ×200 magnification). (*B*) Lung sections were stained with Masson's trichrome on Day 7 after intratracheal LPS or intratracheal water in *Rag-1*$^{-/-}$ mice, *Rag-1*$^{-/-}$ mice + Tregs, and *Rag-1*$^{-/-}$ mice + non-Tregs (images are of representative examples, ×200 magnification, n ⩾ 3 in each group). (*C*) Lung collagen was measured by Sircol assay in all groups on Day 3 and Day 7 after intratracheal LPS (*$P = 0.009$ and $^{\#}P = 0.047$, n ⩾ 4, all groups). (*D*) BAL collagen was measured by Sircol assay in all groups on Day 3, Day 7, and Day 10 after intratracheal LPS (*$P = 0.002$, $^{\dagger}P = 0.003$, $^{\ddagger}P = 0.005$, and $^{\#}P = 0.005$, n ⩾ 3, all groups). (*E*) Lung collagen was positively correlated with the natural log of BAL collagen ($r = 0.73$, $P < 0.001$, using Pearson product moment correlation).

antibody (Rockland Chemicals, Gilbertsville, PA) with a PE–Texas red–conjugated streptavidin secondary (BD Biosciences) and APC-conjugated anti-CXCL12 (R&D Systems).

Statistical Analysis

Mortality was analyzed by χ^2 test. Markers of injury were compared using the Student *t* test or Mann-Whitney rank sum test. Multiple groups were compared using one-way ANOVA or one-way ANOVA on ranks. Pairwise comparisons were performed using the Student *t* test with the Bonferroni correction or the Dunn test. Lung CXCL12 was compared with fibrocyte numbers, using linear regression analysis on log-transformed data. Statistical analysis was performed using Sigmaplot 11.0 (Systat Software, Inc., Chicago, IL).

Further details on adoptive transfers, FACS, the Sircol assay, ELISA, and immunohistochemistry are provided in the online supplement.

RESULTS

Resolution of LPS-Induced Lung Injury Was Impaired in Lymphocyte-Deficient Mice

After the administration of intratracheal LPS, *Rag-1*$^{-/-}$ mice demonstrated increased mortality when compared with WT mice (Figure 1A). Both WT and *Rag-1*$^{-/-}$ mice lost body weight, but WT mice returned to near-baseline body weight by Day 7, whereas *Rag-1*$^{-/-}$ mice demonstrated persistently decreased body weight (Figure 1B). Both WT and *Rag-1*$^{-/-}$ mice exhibited similar degrees of lung injury on Day 3, as indicated by BAL cell count (Figure 1C) and protein (Figure 1D). Both BAL cell counts and protein returned to near baseline values by Day 7 in WT mice, whereas these remained elevated in *Rag-1*$^{-/-}$ mice (Figures 1C and 1D).

## Adoptive Transfer of Tregs Restored Normal Resolution of Lung Injury

After magnetic bead isolation, Tregs (CD4$^+$CD25$^+$ cells; 90% Foxp3$^+$) or non-Tregs (CD4$^+$CD25$^-$ cells) were adoptively transferred into intratracheal LPS–exposed Rag-1$^{-/-}$ mice. The adoptive transfer of Tregs but not non-Tregs into Rag-1$^{-/-}$ mice 1 hour after intratracheal LPS restored the normal resolution of lung injury, as evidenced by significantly improved mortality, increased body weight, and decreased markers of lung injury (Figure 1). Adoptive transfer was confirmed using flow cytometry to identify Tregs (CD4$^+$Foxp3$^+$ cells) or non-Tregs (CD4$^+$Foxp3$^-$ cells) in the BAL, lungs, and spleens of Rag-1$^{-/-}$ mice (see Figure E1 in the online supplement). These results confirm the critical role of Tregs in the normal resolution of LPS-induced lung injury, and establish the pattern of responses against which all other parameters are compared.

LPS-Induced Fibroproliferation Resolved in the Presence of Tregs

We assessed fibroproliferation by histology as well as by measuring collagen concentrations in the BAL and lungs of WT mice, Rag-1$^{-/-}$ mice, and Rag-1$^{-/-}$ mice that received Tregs or non-Treg CD4$^+$ cells. Collagen deposition and interstitial thickening were evident in both WT and Rag-1$^{-/-}$ mice on Day 3 after LPS injury. However, histologic changes resolved in WT mice by Day 7, whereas Rag-1$^{-/-}$ mice demonstrated persistent interstitial thickening and collagen deposition (Figure 2A). The adoptive transfer (AT) of Tregs but not non-Tregs into Rag-1$^{-/-}$ mice led to the histopathologic resolution of fibroproliferation (Figure 2B). Lung collagen concentrations (using the Sircol assay) increased in both WT and Rag-1$^{-/-}$ mice on Day 3, but remained persistently elevated in Rag-1$^{-/-}$ mice on Day 7 after LPS injury (Figure 2C). The AT of Tregs into Rag-1$^{-/-}$ mice reduced lung collagen concentrations to baseline by Day 7. Lung collagen remained significantly elevated in mice receiving non-Tregs, compared with both WT mice and mice that received Tregs. We also examined BAL fluid to see if collagen concentrations were altered in the alveolar compartment. WT and Rag-1$^{-/-}$ mice exhibited increased collagen in BAL fluid on Day 3 after LPS injury (Figure 2D). BAL collagen concentrations returned to baseline in WT mice, but remained elevated in Rag-1$^{-/-}$ mice up to Day 10 after LPS injury, compared with WT mice. The AT of Tregs, but not non-Tregs, into Rag-1$^{-/-}$ mice returned BAL collagen concentrations to baseline. BAL collagen was positively correlated with lung collagen, suggesting that fibroproliferation as measured in the alveolar compartment reflects overall lung fibroproliferation ($r = 0.73$, $P < 0.001$ by Pearson product moment correlation; Figure 2E). These data strongly support a vital role for Tregs in the resolution of fibroproliferation after LPS injury.

Tregs Reduced Lung and BAL Fibrocyte Number after ALI

Fibrocytes are circulating progenitor cells that can be recruited to sites of injury to become collagen-producing myofibroblasts. Fibrocytes can be identified according to the expression of the surface markers CD45 and CXCR4, and the intracellular expression of collagen-1 (Col-I) (12, 13) (Figure E2A). Using flow cytometry, we measured fibrocyte numbers in the lungs (Figure 3A) and BAL (Figure 3B) of WT and Rag-1$^{-/-}$ mice after LPS injury. Despite similar increases in both lung and BAL fibrocytes on Day 3 after LPS injury, fibrocytes were persistently elevated in Rag-1$^{-/-}$ mice compared with WT mice on Day 7. The AT of Tregs, but not non-Tregs, into Rag-1$^{-/-}$ mice returned
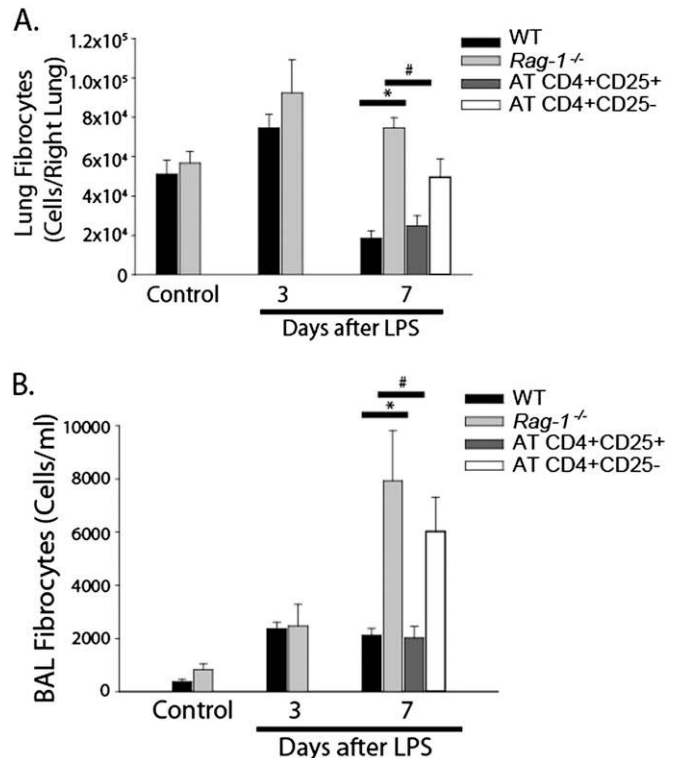

Figure 3. Fibrocytes were initially increased after LPS injury, and were reduced in the presence of Tregs. (A) Lung fibrocyte number was determined using flow cytometry to identify CD45$^+$Col-1$^+$CXCR4$^+$ cells in lung single-cell suspensions in all groups on Day 3 and Day 7 after intratracheal LPS (*$P = 0.007$ and #$P = 0.017$, n ⩾ 6, all groups). (B) BAL fibrocyte number was determined using flow cytometry to identify CD45$^+$Col-1$^+$CXCR4$^+$ cells in BAL single-cell suspensions on Day 3 and Day 7 after intratracheal LPS (*$P < 0.05$ and #$P < 0.05$, n ⩾ 4, all groups).

both lung and BAL fibrocytes to WT concentrations. These results suggest that one of the mechanisms by which Tregs resolve fibroproliferation after LPS injury occurs through a reduction in lung fibrocyte number.

Epithelial-Derived CXCL12 Led to Fibrocyte Recruitment after LPS Injury

CXCL12 (or stromal cell-derived factor 1 [SDF-1]) is the most potent ligand for the CXCR4 receptor (expressed on fibrocytes), and is an important stimulus for fibrocyte recruitment to the lung after injury (12). We used immunohistochemistry to stain for CXCL12 in lung sections from WT and Rag-1$^{-/-}$ mice that had received either intratracheal water or LPS. CXCL12 was increased in both WT and Rag-1$^{-/-}$ mice on Day 3 after lung injury. On Day 7, CXCL12 returned to near baseline in WT mice, but Rag-1$^{-/-}$ mice demonstrated persistently elevated CXCL12 expression. At higher magnifications, epithelial cells appeared to comprise the predominant cell type that stained positive for CXCL12 after LPS injury (Figure 4A).

To confirm that epithelial cells were the major source of CXCL12 expression after LPS injury, we used multicolor flow cytometry to characterize intracellular CXCL12 expression in various cell populations in both WT and Rag-1$^{-/-}$ mice on Day 7 after LPS injury. We used biotinylated CD31 and CD45 antibodies with a V450-conjugated streptavidin to distinguish endothelial cells (CD31$^+$) and leukocytes (CD45$^+$) from other cell populations.
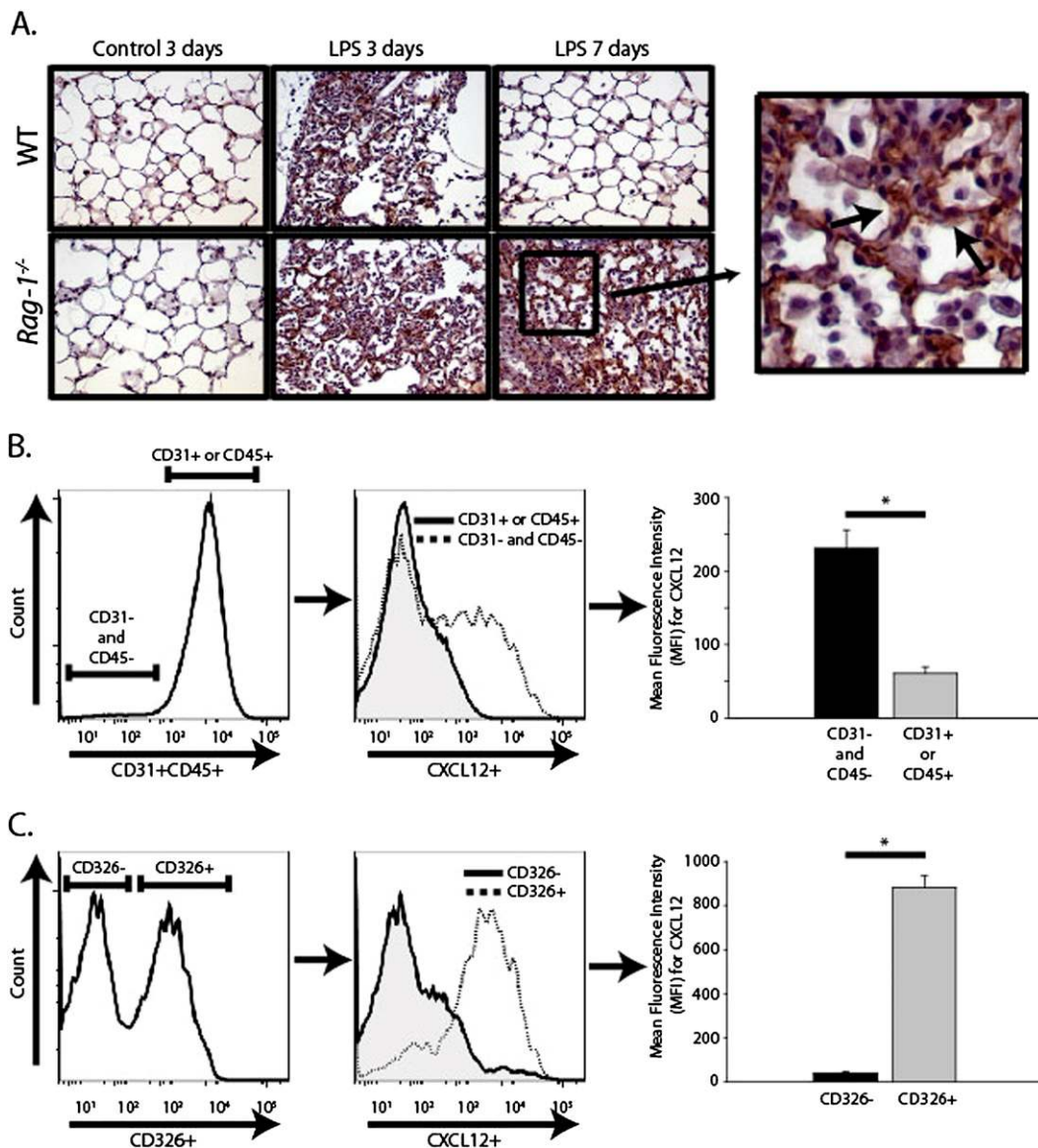
Figure 4. Epithelial cells were an important source of CXCL12 after LPS-induced lung injury. (*A*) Lung sections were stained for CXCL12 by immunohistochemistry on Day 3 and Day 7 after intratracheal LPS. Epithelial cells (*arrows*) appeared to be the predominant source of CXCL12 staining (images are of representative examples, ×200 magnification, *n* ≥ 2 mice in each group; *inset*, ×400 magnification). (*B*) Single-cell suspensions were prepared from the lungs of WT and *Rag-1*$^{-/-}$ mice on Day 7 after intratracheal LPS or sterile water. Cells were stained with biotinylated anti-CD31 and anti-CD45, followed by V450-conjugated streptavidin as well as allophycocyanin-conjugated (APC)–Cy7–conjugated anti-CD326. Cells were fixed, permeabilized, and stained with APC-conjugated anti-CXCL12. Cells were gated for CD31 and CD45 positivity, and CXCL12 expression was examined by mean fluorescence intensity (MFI) (*$P < 0.001$, $n = 16$ mice). (*C*) CD31$^-$CD45$^-$ cells were gated for CD326 (epithelial cell adhesion molecule [EpCAM]) expression to identify epithelial cells. CXCL12 expression in CD326$^+$ and CD326$^-$ cells was examined by MFI (*$P < 0.001$, $n = 16$ mice).

CD31(−)CD45(−) cells exhibited a fourfold higher level of expression of CXCL12 by mean fluorescence intensity, suggesting that leukocytes and endothelial cells were not important sources of elevated lung CXCL12 expression after LPS injury (Figure 4B). Further examination of the CD31(−)CD45(−) cells revealed that the subset of cells that stained positive for the pan-epithelial marker, CD326, demonstrated a 22-fold higher level of CXCL12 expression compared with CD326-cells (Figure 4C). CD326, or epithelial cell adhesion molecule (EpCAM), was recently used to characterize human lung epithelial cell subsets (22). This provides strong evidence that epithelial cells were the primary source of the increased CXCL12 expression after LPS injury.

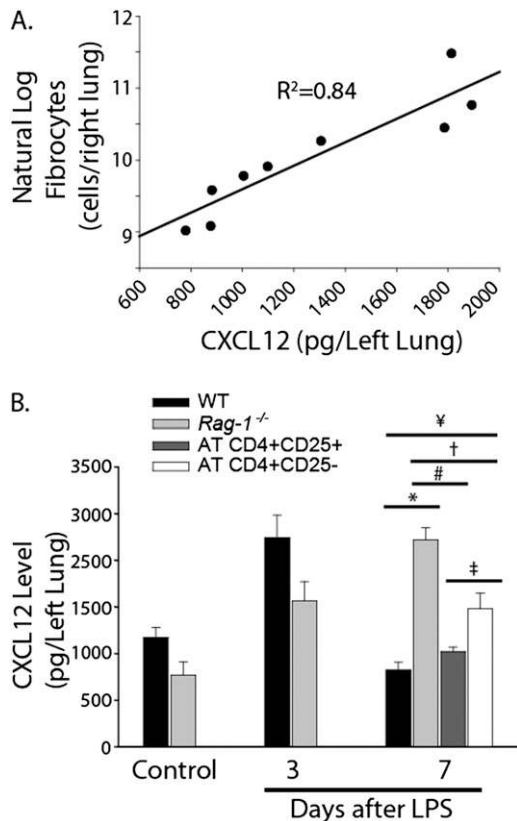## Tregs Reduced Lung CXCL12 Concentrations after LPS Injury

To better quantify CXCL12 expression and to determine the role of Tregs in the regulation of CXCL12 after LPS injury, we measured lung CXCL12 concentrations by ELISA in WT mice, *Rag-1*$^{-/-}$ mice, and *Rag-1*$^{-/-}$ mice that received Tregs and non-Treg CD4$^+$ lymphocytes. CXCL12 concentrations predicted lung fibrocyte numbers according to linear regression analysis ($r = 0.91$, $R^2 = 0.84$, $P < 0.001$; Figure 5A), consistent with the

established role of CXCL12 as a chemokine for fibrocyte recruitment.

CXCL12 concentrations were significantly increased in both *Rag-1*$^{-/-}$ and WT mice on Day 3 after LPS injury (Figure 5B). CXCL12 concentrations returned to baseline by Day 7 in WT mice, but remained significantly elevated in *Rag-1*$^{-/-}$ mice. The AT of Tregs into *Rag-1*$^{-/-}$ mice reduced CXCL12 concentrations to baseline. The AT of non-Tregs into *Rag-1*$^{-/-}$ mice led to a slight reduction in CXCL12 concentrations, but CXCL12 remained significantly elevated compared with the *Rag-1*$^{-/-}$ control (92% higher), WT Day 7 LPS (80% higher), and AT Treg groups (45% higher). These data suggest that Tregs reduced the epithelial expression of CXCL12 after LPS injury, leading to decreased fibrocyte recruitment and the resolution of fibroproliferation.

Blockade of the CXCR4 Receptor with AMD3100 Decreased Fibroproliferation by Reducing Fibrocyte Recruitment to the Lung

To better understand the role of the CXCL12–CXCR4 axis in ALI fibroproliferation, we used the chemical AMD3100 to block the CXCR4 receptor. AMD3100 is a nonpeptide

Figure 5. Adoptive transfer of Tregs reduced lung CXCL12 concentrations. CXCL12 concentrations were measured in whole-lung homogenates by ELISA. (*A*) CXCL12 concentrations were positively correlated with the natural log of lung fibrocyte numbers ($r = 0.91$, $R^2 = 0.84$, $P < 0.001$, using linear regression on log-transformed data; $n = 9$ total mice where CXCL12 concentrations and lung fibrocyte numbers were available from the same time point: 5 $Rag-1^{-/-}$ AT non-Tregs, 3 $Rag-1^{-/-}$ AT-Tregs, and 1 WT Day 7 LPS). (*B*) CXCL12 concentrations were determined by ELISA on whole-lung homogenates on Day 3 and day 7 after intratracheal LPS (*$P < 0.001$, #$P < 0.001$, †$P = 0.009$, ¥$P = 0.01$, and ‡$P = 0.036$, $n \geq 4$, all groups).

antagonist of CXCR4 that inhibits the binding and function of CXCL12 with high affinity and specificity (23, 24). $Rag-1^{-/-}$ mice received intratracheal LPS on Day 0, followed by daily intraperitoneal injections of either AMD3100 (200 μg in 250 μl) or sterile PBS (250 μl), starting on Day 0. Mortality was no different between $Rag-1^{-/-}$ mice that received AMD3100 and $Rag-1^{-/-}$ mice that received PBS (25% and 27%, respectively). Peak weight loss was measured at nearly 25% of initial body weight in both groups, and was similar to the peak weight loss observed in WT and $Rag-1^{-/-}$ mice that received LPS alone (Figure 6A). BAL protein and cell counts were not significantly different between groups (Figure E4). In $Rag-1^{-/-}$ mice, CXCR4 receptor blockade with AMD3100 decreased lung fibrocytes (Figure 6B), histologic fibrosis (Figure 6C), and lung collagen (Figure 6D) on Day 7 after intratracheal LPS. Lung CXCL12 concentrations were no different between $Rag-1^{-/-}$ mice that received AMD3100 or PBS (Figure 6E), indicating that the difference in fibrocyte numbers between groups was attributable to the CXCR4 receptor blockade rather than a decrease in CXCL12 expression. WT animals receiving intratracheal LPS and daily injections of AMD3100 did not exhibit reduced inflammation on Day 4 after lung injury, as measured by BAL protein (Figure E4A) and cell count (Figure E4B), demonstrating

that AMD3100 does not markedly alter the inflammatory response to LPS. These results provide further evidence that fibrocytes recruited along the CXCL12–CXCR4 axis play an important role in fibroproliferation after LPS injury, because blocking CXCR4 reduces fibrocyte numbers, collagen deposition, and fibrosis in $Rag-1^{-/-}$ mice.

DISCUSSION

Fibroproliferation is part of the normal repair response to ALI (5). This repair begins early, as evidenced by increased markers of collagen turnover in the BAL fluid of patients with ARDS within the first days of diagnosis (25, 26). However, excessive fibroproliferation is associated with worse outcomes, because patients develop decreased lung compliance and increased dead space, and require prolonged mechanical ventilation (4, 6, 8). Despite its importance to patient outcomes, the factors that underlie fibroproliferation in ALI remain poorly understood.

Tregs down-regulate lung inflammation in a number of contexts (27–30). Because the fibroproliferative phase of ARDS is characterized by persistent interstitial inflammation (4), Tregs might be expected to reduce fibroproliferation, perhaps by dampening the inflammation that leads to myofibroblast accumulation and collagen deposition (31). Patients with IPF and collagen vascular disease–associated pulmonary fibrosis demonstrate a reduced number and function of Tregs in BAL fluid and peripheral blood, compared with controls, suggesting that Tregs may play a role in fibroproliferation (27). However, conflicting data in the literature have emerged regarding Tregs in animal models of pulmonary fibrosis (31–34), suggesting that the role of Tregs in regulating fibroproliferation may be both context-specific and site-specific. To our knowledge, the role of Tregs in the fibroproliferative response to ALI has not been described.

Our data support a novel role for Tregs in accelerating the resolution of fibroproliferation by decreasing fibrocyte recruitment to the lung after injury. Fibrocytes are $CD45^+Col-1^+CXCR4^+$ bone marrow–derived cells that migrate to sites of injury to become collagen-producing myofibroblasts (12, 13). Fibrocytes play an important role in bleomycin models of pulmonary fibrosis (10, 15). They are present in the BAL fluid and blood of patients with IPF, and increased fibrocyte numbers predict exacerbations (14, 16). Fibrocytes are also found in the BAL fluid of patients with ARDS, and are associated with increased mortality (17, 18).

CXCL12 is constitutively expressed in a number of tissues, and binds to the CXCR4 receptor on fibrocytes to promote migration (12, 14, 35–37). The significant association between lung CXCL12 concentrations and fibrocyte numbers in our model supports previous studies that demonstrated the importance of CXCL12 in fibrocyte recruitment (12, 13). CXCL12 secretion is induced in rat Type II epithelial cells after scratch injury, implicating the epithelium as an important source of CXCL12 (38). In patients with IPF, epithelial cells expressed CXCL12 at higher concentrations than in control samples (14). Epithelial CXCL12 increases in both murine and human lungs after ALI (39). Our data indicate that $CD326^+$ epithelial cells are the most abundant source of CXCL12 expression after LPS-induced ALI.

Our study shows that Tregs facilitate the resolution of ALI fibroproliferation by decreasing lung CXCL12 concentrations, leading to a reduction in lung fibrocytes and a decrease in collagen deposition and fibrosis. Tregs might alter lung CXCL12 concentrations in a number of ways. Because epithelial cells are the most abundant source of CXCL12 after LPS injury, Tregs likely modulate epithelial CXCL12 expression. Treg–epithelial interactions were previously described. For example, crosstalk between Tregs and the intestinal or retinal epithelium is an
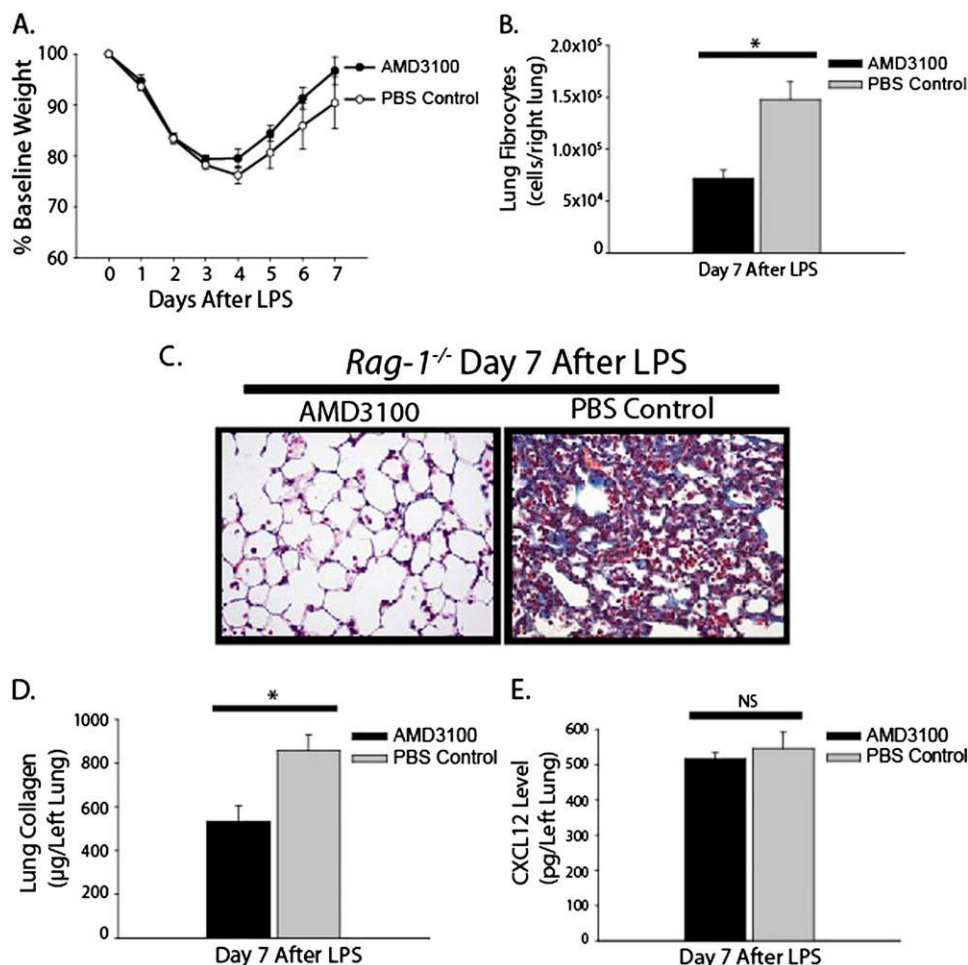
Figure 6. Blockade of the CXCR4 receptor with AMD3100 reduced fibroproliferation in *Rag-1*$^{-/-}$ mice by reducing fibrocyte recruitment to the lung after LPS injury. (*A*) Weight loss presented as percentage of baseline weight after intratracheal LPS for *Rag-1*$^{-/-}$ mice that received intraperitoneal AMD3100 or intraperitoneal PBS. (*B*) Lung fibrocyte number was determined using flow cytometry to identify CD45$^+$Col-1$^+$ CXCR4$^+$ cells in lung single-cell suspensions in both groups on Day 7 after intratracheal LPS (*$P = 0.001$; n $\geqslant$ 7, all groups). (*C*) Lung sections were stained with Masson's trichrome to highlight collagen deposition on Day 7 after intratracheal LPS in both groups (images are of representative examples; magnification, ×200). (*D*) Lung collagen was measured by Sircol assay in both groups on Day 7 after intratracheal LPS (*$P = 0.012$; n $\geqslant$ 5, all groups). (*E*) CXCL12 concentrations were measured in whole-lung homogenates by ELISA in both groups on Day 7 after intratracheal LPS (n $\geqslant$ 5, all groups).

important determinant of both immune tolerance and Treg proliferation (40–42). Type II alveolar epithelial cells (AECs) can present antigen to naive T cells through the Class II major histocompatibility complex (MHC), and induce the proliferation of Tregs, in part through a transforming growth factor (TGF)–β–dependent mechanism (43). In the context of our model, Tregs might down-regulate AEC CXCL12 expression in a contact-dependent manner. For example, the engagement of Class II MHC receptors on epithelial cells by Tregs might lead to a change in CXCL12 expression. Furthermore, a contact-independent interaction might occur between Tregs and AECs, perhaps through the release of cytokines that decrease AEC CXCL12 production. Tregs may also exert their effects on AECs through a third cell type. For example, our group has shown that Tregs decrease the macrophage production of TNF-α in a contact-dependent manner (19). An alteration in macrophage phenotype might then lead to a decrease in AEC CXCL12 production. Future experiments will seek to identify the specific interactions between Tregs and epithelial cells that govern overall CXCL12 expression and subsequent fibrocyte recruitment in the context of ALI.

The reduction in lung collagen concentrations and fibrocytes in *Rag-1*$^{-/-}$ mice after CXCR4 receptor blockade with AMD3100 provides further evidence that the CXCL12–CXCR4 axis is critical in fibrocyte-mediated ALI fibroproliferation, and is consistent with previous studies that demonstrated a reduction in lung fibrosis in bleomycin models after CXCR4 or CXCL12 blockade (12, 24). The lack of a significant difference in BAL cell count, protein, and lung CXCL12 concentrations between AMD3100-treated

Rag-1$^{-/-}$ mice and PBS control mice, as well as the similar peak weight loss in each group, indicate that the difference in fibroproliferation did not result from reduced inflammation after LPS injury, and was instead attributable to the decrease in fibrocyte number. The uncoupling of inflammation and fibroproliferation in this context further supports the notion that the reduction in fibroproliferation seen in our Treg AT experiments was not solely the result of a Treg-dependent decrease in inflammation, but was in part attributable to a reduction in fibrocyte recruitment. Given the potential difficulties of using Tregs as therapy for human autoimmune and inflammatory diseases (44), the pharmacologic manipulation of downstream pathways such as the CXCL12–CXCR4 axis may provide attractive targets for ALI treatment in the future.

Factors in addition to fibrocyte recruitment along the CXCL12–CXCR4 axis likely modulate fibrocyte numbers and subsequent fibroproliferation in the context of ALI. For example, Tregs could influence both fibrocyte proliferation and survival. Moreover, fibrocyte number alone clearly does not explain the differences in lung collagen concentrations seen between our experimental groups, as evidenced by the finding that lung fibrocytes were lower in WT mice on Day 7 after LPS compared with water control mice, yet lung collagen concentrations were slightly higher in the Day 7 WT mice. The collagen-producing capacity of fibrocytes likely depends in part on their surrounding environment. In addition, our flow cytometry markers may not have identified fibrocytes as they transitioned to a myofibroblast phenotype. In culture, fibrocytes lose the expression of CD34 and CD45 as they transition to a collagen-producing cell (45), making them potentially difficult to track once they transition to a myofibroblast phenotype *in vivo*. Future

experiments will seek to examine differences in fibrocyte proliferation, survival, and collagen-producing capacity in the context of ALI fibroproliferation, and to refine the tracking of fibrocytes after they transition to a myofibroblast phenotype.

Our study contains other important limitations. Although the purity of our bead-sorted Tregs used in adoptive transfers was consistently greater than 85%, some $CD4^-CD25^+$ cells were included in our ATs. Although these represented on average less than 7.5% of overall transferred cells, they may have contributed to the reduction in fibroproliferation. In addition to Tregs, CD25 can be expressed in $CD8^+$ T cells, activated B cells, and (at lower concentrations) monocytes. Almost all of the $CD4^-CD25^+$ cells demonstrated a low forward and side scatter by flow cytometry, suggesting they were likely lymphocytes (data not shown). Our group has shown that the AT of splenocytes from CD4-depleted spleens into $Rag-1^{-/-}$ mice does not resolve LPS-induced lung injury. Furthermore, the AT of $CD8^+$ lymphocytes into $Rag-1^{-/-}$ mice dose not result in the resolution of lung injury (19). This suggests that $CD4^-CD25^+$ cells do not play a role in reducing fibroproliferation in our model. Treg depletion with the CD25-specific antibody PC61 impairs the resolution of lung injury, further emphasizing the critical role of Tregs in our model of lung injury (19).

The small but significant decrease in CXCL12 concentrations after the AT of non-Tregs into $Rag-1^{-/-}$ mice might suggest that $CD4^+$ lymphocytes other than Tregs play a role in reducing fibroproliferation. However, this small reduction in CXCL12 did not result in decreased biochemical or histologic markers of fibroproliferation. The decrease in CXCL12 may also have been the result of a small number of Tregs present in the non-Treg ATs, either from bead separation impurities or from the induction of naive $CD4^+$ lymphocytes into Tregs (46). Although we did not find significant numbers of Tregs in the lungs of $Rag-1^{-/-}$ mice that received non-Tregs on Day 7 after AT, the number of Tregs required to reduce CXCL12 concentrations and the kinetics of this response remain unknown.

Although our study highlighted an important role for fibrocytes in ALI fibroproliferation, it did not address the potential contributions of other cell types to the activated myofibroblast pool. Resident fibroblasts were shown to be an important source of collagen deposition and fibrosis in both animal and human models. Recently an interest has emerged in the concept of epithelial-to-mesenchymal transition, whereby epithelial cells may either transform into collagen-producing myofibroblasts or secrete profibrotic growth factors and cytokines that can lead to fibroproliferation (10, 47). Future experiments will seek to define the role of these cell types in our injury model.

In conclusion, we report on a unique role for Tregs in the resolution of ALI fibroproliferation through the control of fibrocyte recruitment to the lung after LPS injury. A better understanding of the role of Tregs in the fibroproliferative response will potentially uncover pathways that might lead to new diagnostic and therapeutic options in patients with ALI.

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