Tumor Necrosis Factor- α Accelerates the Resolution of Established Pulmonary Fibrosis in Mice by Targeting Profibrotic Lung Macrophages

Elizabeth F. Redente¹, Rebecca C. Keith³, William Janssen^{2,3}, Peter M. Henson^{1,3,4}, Luis A. Ortiz⁶, Gregory P. Downey^{2,3,4}, Donna L. Bratton¹, and David W. H. Riches^{1,3,4,5}

¹Program in Cell Biology, Department of Pediatrics, and ²Department of Medicine, National Jewish Health, Denver, Colorado; ³Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, ⁴Integrated Department of Immunology, and ⁵Department of Pharmacology, University of Colorado School of Medicine, Aurora, Colorado; and ⁶Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh Pennsylvania

Abstract

Idiopathic pulmonary fibrosis (IPF) is a relentless, fibrotic parenchymal lung disease in which alternatively programmed macrophages produce profibrotic molecules that promote myofibroblast survival and collagen synthesis. Effective therapies to treat patients with IPF are lacking, and conventional therapy may be harmful. We tested the hypothesis that therapeutic lung delivery of the proinflammatory cytokine tumor necrosis factor (TNF)- α into wild-type fibrotic mice would reduce the profibrotic milieu and accelerate the resolution of established pulmonary fibrosis. Fibrosis was assessed in bleomycin-instilled wild-type and TNF- $\alpha^{-/-}$ mice by measuring hydroxyproline levels, static compliance, and Masson's trichrome staining. Macrophage infiltration and programming status was assessed by flow cytometry of enzymatically digested lung and *in situ* immunostaining. Pulmonary delivery of TNF- α to wild-type mice with established pulmonary fibrosis was found to reduce their fibrotic burden, to improve lung function and architecture, and to

reduce the number and programming status of profibrotic alternatively programmed macrophages. In contrast, fibrosis and alternative macrophage programming were prolonged in bleomycin-instilled TNF- $\alpha^{-/-}$ mice. To address the role of the reduced numbers of alternatively programmed macrophages in the TNF- α -induced resolution of established pulmonary fibrosis, we conditionally depleted macrophages in MAFIA (MACrophage Fas-Induced Apoptosis) mice. Conditional macrophage depletion phenocopied the resolution of established pulmonary fibrosis observed after therapeutic TNF- α delivery. Taken together, our results show for the first time that TNF- α is involved in the resolution of established pulmonary fibrosis via a mechanism involving reduced numbers and programming status of profibrotic macrophages. We speculate that pulmonary delivery of TNF- α or augmenting its signaling pathway represent a novel therapeutic strategy to resolve established pulmonary fibrosis.

Keywords: tumor necrosis factor- α ; pulmonary fibrosis; macrophages; inflammation

Idiopathic pulmonary fibrosis (IPF) is a restrictive, interstitial lung disease in which progressive fibrosis of the alveolar-capillary units leads to respiratory failure and death

(1, 2). Defined histologically by the development of usual interstitial pneumonia, excessive fibroblast/myofibroblast accumulation, and collagen deposition in

fibroblastic foci, patients with IPF exhibit a median survival of only 2 to 3 years after diagnosis (2, 3). Combined with the absence of therapies to control or reverse

(Received in original form September 6, 2013; accepted in final form December 4, 2013)

This work was supported by National Institutes of Health Public Health Service grants HL068628 and HL114754 (D.W.H.R.), HL109517 (W.J.), HL088138 and HL081151 (P.M.H.), HL034303 and AI058228 (D.L.B.), ES010859 (L.A.O.), and HL090669 (G.P.D.); by the Chronic Granulomatous Disease Society and Catherine Kramer Foundation (D.L.B.); and by a Ruth L. Kirschstein National Research Service Award (F32HL095274) from the National Heart Lung and Blood Institute, a Viola Vestal Coulter Foundation Fellowship, and a Natalie V. Zucker Research Center for Women Scholars Grant (E.F.R.).

Author Contributions: E.F.R. participated in study conception, experimental design, data acquisition, analysis, interpretation, and manuscript preparation. R.C.K. participated in data acquisition, analysis, and manuscript preparation. W.J., P.M.H., L.A.O., G.P.D., and D.L.B. participated in study design, data interpretation, and manuscript preparation. D.W.H.R. participated in study conception, experimental design, data interpretation, and manuscript preparation.

Correspondence and requests for reprints should be addressed to David W. H. Riches, Ph.D., Program in Cell Biology, Department of Pediatrics, Iris and Michael Smith Building Room A549, National Jewish Health, 1400 Jackson Street, Denver, CO 80206. E-mail: richesd@NJHealth.org

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 50, Iss 4, pp 825–837, Apr 2014

Copyright © 2014 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2013-0386OC on December 10, 2013

Internet address: www.atsjournals.org

Clinical Relevance

Pulmonary fibrosis is an intractable parenchymal lung disease of high morbidity and mortality. Although much has been learned about the mechanisms that contribute to the development of fibrosis, effective therapies to resolve established fibrosis are lacking. Building on previous studies highlighting the importance of profibrotic, alternatively programmed macrophages in the development of pulmonary fibrosis, this study demonstrates that therapeutic lung delivery of the proinflammatory cytokine TNF- α resolves established pulmonary fibrosis in a mouse model by mitigating the profibrotic activity of lung macrophages. Our findings raise the possibility that pulmonary delivery of TNF- α or other approaches that target the profibrotic activity of macrophages may represent novel approaches to resolve established fibrosis.

the fibrotic process and the generally held view that the disease is irreversible, the outlook for patients with IPF is undeniably bleak. Thus, there is a pressing need for effective therapies.

Many factors contribute to the development and persistence of fibrosis in the lung parenchyma of patients with IPF. Especially important is the development of a profibrotic-, transforming growth factor (TGF)- β 1-, and growth factor-rich microenvironment that supports fibroblast migration and recruitment, promotes their proliferation and differentiation into myofibroblasts, and prevents them from undergoing apoptosis (4–6). Together, these microenvironmental signals facilitate fibroblast and myofibroblast accumulation and persistence in the lungs of patients with IPF, where they continue to synthesize fibronectin, collagen, and other components that constitute fibrotic lung tissue (7). Resident and infiltrating inflammatory lung macrophages contribute to the development of pulmonary fibrosis through their ability to create and sustain the profibrotic lung microenvironment (4, 8–10). Current concepts suggest that macrophages become programmed to express a continuum of gene expression patterns that enable them to

participate in a spectrum of varied settings, including inflammation, host defense against pathogenic bacteria, and wound healing and repair (11, 12). So-called “alternatively programmed” macrophages (11) express profibrotic genes such as TGF- β 1, IGF-I, PDGF, and arginase I (9, 11, 13) and have been linked to the development of pulmonary fibrosis (8, 14). In contrast, “classically programmed,” inducible nitric oxide synthase 2 (NOS2)-expressing macrophages develop in response to proinflammatory cytokines and Toll-like receptor (TLR) agonists such as IFN- γ , tumor necrosis factor (TNF)- α , LPS, and poly [I:C] and are typically seen in acute and chronic lung inflammation and in host defense (15, 16). Although the relationship between these distinct programming states is complex, it is clear that macrophages adapt to ongoing changes in their microenvironment by altering their patterns of gene expression (17–20). Taken together, these findings raise the question of whether profibrotic alternative macrophage programming can be mitigated to reduce their capacity to support fibrosis and potentially resolve established fibrosis.

We hypothesized that decreasing the profibrotic lung microenvironment by therapeutic delivery of the proinflammatory cytokine TNF- α might represent an effective way to reverse established pulmonary fibrosis and accelerate resolution toward normal structure and function. We also hypothesized that this shift would reduce profibrotic macrophage numbers and their alternative programming status. We tested these hypotheses using the bleomycin model of pulmonary fibrosis in mice. Some of the results of these studies have been previously reported in the form of an abstract (21).

Materials and Methods

Animals

Male C57BL/6J, TNF- α -deficient (B6.129S-Tnf^{tm1Gkl/J}) and Macrophage Fas-Induced Apoptosis (MAFIA) [C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J] mice (6–8 wk of age) were purchased from the Jackson Laboratory (Bar Harbor, ME) and studied as approved by the National Jewish Health Institutional Animal Care and Use Committee. Fifteen mice were instilled for each experimental treatment condition.

Induction, Measurement, and Treatment of Pulmonary Fibrosis

Pulmonary fibrosis was initiated by the intratracheal instillation of 50 μ l of bleomycin (3 U/kg) (TEVA Pharmaceuticals, North Wales, PA) as previously described (22). Control mice were instilled with 50 μ l of saline. Fibrosis was assessed by lung measurements of static compliance, collagen (hydroxyproline) levels, and evaluation of Masson's trichrome-stained sections as reported (22). As described in the RESULTS, mice were intratracheally instilled with 50 μ l of recombinant mouse TNF- α (500 ng) (PeproTech, Rocky Hill, NJ) at 3 and 4 weeks and harvested at 5 weeks. No mortality was associated with TNF- α administration.

Depletion of Macrophages in MAFIA Mice

Macrophages were conditionally depleted in MAFIA mice, which express a chimeric FK506 binding protein-Fas death receptor under the *c-fms* intronic enhancer, by the intravenous injection of the synthetic dimerizer AP20187 (10 mg/kg) (Ariad Pharmaceuticals, Inc., Cambridge, MA) reconstituted as previously described (23).

Enzymatic Dispersal of Pulmonary Macrophages and Flow Cytometry

Mice were killed by lethal intraperitoneal injection of Nembutal (Ovation Pharmaceuticals, Paramus, NJ), and the lungs were lavaged (22). Single-cell suspensions were obtained from perfused, enzymatically dispersed lungs (24). Cells were incubated with Mouse BD Fc Block (BD Biosciences, San Diego, CA) for 30 minutes and stained with fluorescently tagged monoclonal antibodies against CD11c, MHCII, Ly6G (eBiosciences, San Diego, CA), CD11b, and F4/80 (BD Pharmingen, San Diego, CA) as described (25). CD206 (Serotec, Raleigh, NC) and NOS2 (BD Biosciences) were used to stain alternatively and classically programmed macrophages, respectively. The mean fluorescence intensities (MFIs) for CD206 and NOS2 were determined for the entire population of CD11c⁺CD11b^{var} and CD11c⁺CD11b⁺ macrophages. Dendritic cells identified as CD11c⁺CD11b^{var}MHCII^{hi}F4/80⁺ were eliminated from macrophages followed by confirmation of being SSC^{lo}. Data were acquired with a LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence Analysis of Macrophage Programming

Immunofluorescence staining of paraffin-embedded sections using antibodies against F4/80 (Serotec), NOS2 (BD Biosciences), and arginase I (Santa Cruz Biotechnology, Santa Cruz, CA) was conducted as previously described (26). Images were acquired on a Zeiss Axioplan 2 epifluorescence microscope and analyzed with Axiovision software (Zeiss, Thornwood, NY). Quantification of fluorescence intensity from 10 images per animal by pixel counting was accomplished using ImageJ software. The total pixel intensity for arginase I or NOS2 in each image was divided by the pixel intensity for F4/80 from the same image and multiplied by 100 to generate a score (% positive macrophages) for each marker. This approach provides a quantitative value for the macrophage population sampled but does not distinguish specific changes occurring in individual cells.

Arginase activity was measured in the middle right lobe as described (27).

Statistics

Data are presented as the mean $\pm$ SEM. Differences between conditions at specific time points were examined using Student's unpaired *t* test. One-way ANOVA with Newman-Keuls *post hoc* analysis was used to compare results from more than two groups, with $P < 0.05$ considered to be significant.

In Vitro Analysis of Bone Marrow Macrophage Programming

Bone marrow macrophages from wild-type mice were prepared and cultured as previously described (13). After 7 days in culture, macrophages were pretreated for 24 hours with rmIL-4 and rmIL-13 (each at 20 ng/ml) (PeproTech) to induce alternative programming. TNF- α (20 ng/ml) (PeproTech) was added for 24 hours and

macrophage programming was assessed by flow cytometry for CD206 and by arginase activity in cell lysates as described (27).

Quantification of TNF- α by ELISA

TNF- α was measured in lung homogenates by ELISA following the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Results

TNF- α Accelerates the Resolution of Established Pulmonary Fibrosis in Mice

To test the hypothesis that therapeutic TNF- α administration accelerates the resolution of established pulmonary fibrosis, we used the single-dose model of intratracheal bleomycin instillation in mice (28). This model leads to a predictable course of alveolar protein leak (Figure 1A)

Figure 1. The time course of fibrosis development and resolution after bleomycin. (A) Total protein concentration in bronchoalveolar lavage fluid (BALF). (B) Quantification of lavaged bronchoalveolar lavage (BAL) cells after bleomycin-instillation. (C) Hydroxyproline levels measured in saline and bleomycin-instilled mice. (D) Static compliance in saline and bleomycin-instilled mice. (E) Mouse lung sections stained with Masson's trichrome. Under normal conditions, collagen deposition (blue) is limited to areas adjacent to airways and large vessels but spreads throughout the lung in response to bleomycin instillation. Original magnification: $\times 20$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

and airspace inflammation (Figure 1B) at 1 to 2 weeks, followed by the development of parenchymal fibrosis between Weeks 2 to 5 as reflected by increased lung hydroxyproline (Figure 1C), decreased static compliance (Figure 1D), and increased lung collagen content as assessed by Masson's trichrome staining (Figure 1E). Between Weeks 5 and 8 the fibrotic response gradually resolved until all fibrotic parameters approach that of saline-instilled control mice by Weeks 6 to 8. TNF- α was measured by ELISA and was undetectable in bronchoalveolar lavage fluid (BALF) at any time point but was detected in lung homogenates for up to 6 days, peaking 12 hours after bleomycin instillation (see Figure E1 in the online supplement).

To determine if therapeutically administered TNF- α accelerates the resolution of established pulmonary fibrosis, we intratracheally instilled mice with recombinant murine TNF- α (500 ng) (29) or saline as a control at Weeks 3 and 4 after bleomycin treatment (i.e., after fibrosis was established) and assessed inflammatory and fibrotic parameters at Week 5 (Figure 2A). Intratracheal delivery of TNF- α reduced lung collagen levels ($P < 0.001$) (Figure 2B) and improved compliance in fibrotic mice ($P < 0.001$) (Figure 2C). TNF- α instillation also reduced BALF protein levels ($P < 0.001$) (Figure 2D) and inflammatory cell numbers ($P < 0.01$) (Figure 2E). Examination of trichrome-stained lung sections confirmed reduced lung collagen in TNF- α -treated mice and an improvement of lung architecture with areas of relatively normal lung tissue juxtaposed to patches of residual fibrosis (Figure 2F). Instillation of TNF- α into the lungs of saline-instilled mice had no effect on lung histology or on the fibrotic and inflammatory endpoints (Figure 2).

Given the therapeutic effect of TNF- α in resolving established fibrosis, we next determined if endogenously produced TNF- α contributed to the resolution of pulmonary fibrosis. Wild-type and TNF- $\alpha^{-/-}$ mice were instilled with bleomycin, and fibrosis was assessed at 3, 6, and 8 weeks. Bleomycin-instilled wild-type and TNF- $\alpha^{-/-}$ mice developed similar changes in lung collagen levels (Figure 3A) and compliance (Figure 3B) at 3 weeks. However, although lung collagen levels and compliance in wild-type mice had returned to control levels by Weeks 6 and 8, collagen levels remained elevated

Figure 2. Intratracheal delivery of tumor necrosis factor (TNF)- α after bleomycin instillation accelerates the resolution of pulmonary fibrosis. (A) Schematic showing the TNF- α treatment regimen given after bleomycin instillation. All mice were harvested at Week 5. (B) TNF- α significantly attenuates the bleomycin-mediated increase in lung hydroxyproline levels (*** $P < 0.001$). (C) Static compliance is significantly reduced after bleomycin instillation and returns to saline levels after TNF- α administration (*** $P < 0.001$). (D and E) Total BALF protein levels (D) and lavaged cells in the BAL (E) are significantly decreased after TNF- α administration (*** $P < 0.001$). (F) Mouse lung sections after TNF- α administration have less collagen staining by Masson's trichrome compared with bleomycin-instilled mice. Bleo = bleomycin; Sal = saline. Original magnification: $\times 20$.

($P < 0.05$) (Figure 3A), and compliance continued to be reduced ($P < 0.05$) (Figure 3B) in $\text{TNF-}\alpha^{-/-}$ mice. $\text{TNF-}\alpha^{-/-}$ mice also exhibited elevated inflammatory cell numbers in BAL ($P < 0.001$) compared with wild-type mice at 3, 6, and 8 weeks (Figure 3C). Examination of trichrome-stained sections confirmed persistent fibrosis, collagen deposition (stained blue), septal thickening, and inflammatory cell infiltration (stained pink) for up to 8 weeks in the lungs of $\text{TNF-}\alpha^{-/-}$ mice, whereas in wild-type mice, inflammation and lung architecture were considerably improved by Weeks 6 and 8 (Figure 3D). Collectively, these findings indicate that therapeutic delivery of $\text{TNF-}\alpha$ accelerated the resolution of established pulmonary fibrosis, whereas genetic deficiency of $\text{TNF-}\alpha$ prolonged the fibrotic response.

$\text{TNF-}\alpha$ Reduces Profibrotic Alternative Macrophage Programming in Fibrotic Lungs

We hypothesized that the mechanism of $\text{TNF-}\alpha$ resolution of fibrosis involved a reduction in profibrotic alternative macrophage programming. To address this hypothesis, pulmonary macrophages were evaluated by flow cytometry, as described (30, 31). Macrophages were identified as $\text{CD11c}^+ \text{CD11b}^{\text{var}} \text{F4/80}^+ \text{Ly6G}^-$, a subset that includes resident alveolar macrophages and recruited alveolar and interstitial inflammatory macrophages, or as $\text{CD11c}^- \text{CD11b}^+ \text{F4/80}^+ \text{Ly6G}^-$, a subset that primarily contains naive interstitial macrophages (Figure 4A). Alternative and classical macrophage programming markers were evaluated by quantifying the MFI of CD206 (mannose receptor) and intracellular NOS2 staining, respectively, in these two distinct subsets. Compared with saline-instilled mice, the number of $\text{CD11c}^+ \text{CD11b}^{\text{var}} \text{F4/80}^+$ macrophages was increased at the peak of fibrosis in bleomycin-instilled mice at Week 3 but returned to baseline levels by Week 6 (Figure 4B, black bars). $\text{CD11c}^- \text{CD11b}^+ \text{F4/80}^+$ macrophage numbers were relatively unaffected by bleomycin instillation (Figure 4B, white bars). Of these subsets, $\text{CD11c}^+ \text{CD11b}^{\text{var}} \text{F4/80}^+$ macrophages alone exhibited alternative programming markers at the peak of fibrosis at 3 weeks, as revealed by increased CD206 expression, which decreased during resolution ($P < 0.05$) (Figure 4C, black bars). NOS2 expression was low in both macrophage subsets and was largely

Figure 3. $\text{TNF-}\alpha^{-/-}$ mice exhibit delayed resolution of bleomycin-induced pulmonary fibrosis. (A) Hydroxyproline levels remained significantly elevated at 6 and 8 weeks after bleomycin in $\text{TNF-}\alpha^{-/-}$ mice compared with wild-type mice ($*P < 0.05$). (B) Static compliance was significantly decreased at 6 and 8 weeks after bleomycin in $\text{TNF-}\alpha^{-/-}$ mice compared with wild-type mice ($*P < 0.05$). (C) Lavaged cells were significantly elevated in $\text{TNF-}\alpha^{-/-}$ mice at 6 and 8 weeks after bleomycin instillation compared with wild-type mice ($***P < 0.001$) and saline controls ($P < 0.001$). (D) Masson's trichrome-stained lung sections from bleomycin-instilled $\text{TNF-}\alpha^{-/-}$ mice exhibit impaired resolution of fibrosis and increased inflammation (pink) compared with bleomycin-instilled wild-type mice. Original magnification: $\times 20$.

insensitive to bleomycin instillation (Figure 4C). We confirmed these findings by confocal microscopy of lung sections stained with fluorescently tagged antibodies against arginase I (another marker of alternative programming), NOS2, and F4/80

(Figure 4D). Figures 4D and 4E shows that the majority of F4/80^+ macrophages in lung sections from bleomycin-instilled mice expressed arginase I and low levels of NOS2. Arginase catalytic activity similarly increased in lung homogenates from fibrotic

Figure 4. (See figure legend on following page)

bleomycin-instilled mice at 3 weeks and decreased toward control levels as fibrosis resolved (Figure 4F). Taken together, these data indicate that alternative programming was increased only in CD11c⁺CD11b^{var}F4/80⁺ lung macrophages during the development of pulmonary fibrosis, whereas NOS2 was basally expressed at low levels by both macrophage subsets.

On the basis of these results, we questioned whether the accelerated resolution of fibrosis in TNF- α -treated mice was associated with alterations in macrophage programming. Fibrotic, bleomycin-instilled mice were given TNF- α at Weeks 3 and 4 and assessed for macrophage programming at Week 5 (Figure 5B). Compared with saline-instilled fibrotic mice, TNF- α treatment reduced the number of CD11c⁺CD11b^{var}F4/80⁺ macrophages ($P < 0.01$) (Figure 5B, black bars) and the expression of CD206 in the remaining cells ($P < 0.01$), whereas NOS2 expression was modestly increased ($P < 0.01$) in this subset (Figure 5C). TNF- α treatment had no effect on the number of CD11c⁺CD11b⁺F4/80⁺ macrophages or their programming status (Figures 5B and 5C). The changes in CD206 and NOS2 expression (Figure 5B) in the CD11c⁺CD11b^{var}F4/80⁺ subset occurred in a single population of dual-positive cells that expressed variable levels of both markers. Immunostaining of lung sections for F4/80, arginase I, and NOS2 confirmed the down-regulation in macrophage arginase I expression and the reciprocal increase in NOS2 expression in TNF- α -treated fibrotic mice compared with saline-instilled fibrotic mice (Figure 5C). Analysis of pixel density also revealed an increase in NOS2 expressing F4/80⁺ macrophages after treatment with TNF- α , consistent with the flow cytometry data ($P < 0.001$) (Figure 5D). Measurements of lung arginase activity also confirmed that TNF- α instillation reduced arginase levels ($P < 0.05$) (Figure 5E).

Because TNF- α accelerates the resolution of established fibrosis in conjunction with reduced macrophage numbers and alternative programming status, genetic TNF- α deficiency would be predicted to prolong alternative programming in the persistently fibrotic lungs of TNF- α ^{-/-} mice. Immunostaining of lung sections from bleomycin-instilled wild-type and TNF- α ^{-/-} mice confirmed the predominant alternative macrophage programming response at 3 weeks in both

strains (Figure 6A). However, unlike wild-type mice that exhibited a decline in arginase I staining to basal levels by Week 8, greater than 75% of TNF- α -deficient, F4/80⁺ lung macrophages continued to express arginase I at 6 and 8 weeks (Figure 6B, *blue bars*). Levels of NOS2 expression (Figure 6A) and the percentage of NOS2-positive cells ($P = 0.912$) (Figure 6B, *green bars*) were unaffected by the absence of TNF- α . Elevated lung arginase activity confirmed the persistence of this alternative macrophage programming-associated enzyme in the lungs of TNF- α ^{-/-} mice (Figure 6C).

To determine if TNF- α directly affected macrophage programming, wild-type bone marrow-derived macrophages were alternatively programmed *in vitro* with recombinant IL-4 and IL-13. The alternatively programmed macrophages were then incubated with recombinant murine TNF- α and analyzed for changes in CD206 expression and arginase activity. Figure E2 shows that TNF- α significantly reduced the expression of CD206 ($P < 0.001$) and arginase activity ($P < 0.01$), suggesting that TNF- α qualitatively reduces alternative macrophage programming.

Taken together, our findings indicate that the accelerated resolution of established fibrosis occurring in the presence of TNF- α is accompanied by a specific reduction in CD11c⁺CD11b^{var}F4/80⁺ macrophages that have decreased expression of alternative macrophage programming markers and

increased expression of NOS2. In contrast, the impaired resolution of fibrosis seen in TNF- α -deficient mice was associated with the persistence of alternatively programmed macrophages and a failure to up-regulate NOS2. Furthermore, TNF- α was able to directly reduce the expression of the alternative macrophage programming marker CD206 in bone marrow-derived macrophages in a manner similar to what was observed *in vivo*.

Loss of Inflammatory Lung Macrophages Promotes the Resolution of Established Pulmonary Fibrosis

We next addressed the importance of the loss of alternatively programmed lung macrophages in the resolution of fibrosis by conditionally depleting macrophages after fibrosis had been established. MAFIA mice were intratracheally instilled with bleomycin and monocytes, and macrophages were depleted by intravenous delivery of AP20187 between Weeks 2 to 3.5. Fibrosis and inflammatory indices were then assessed at Week 4 (Figure 7A). Fibrotic, bleomycin-instilled MAFIA mice treated with vehicle exhibited the expected increase in CD11c⁺CD11b^{var}F4/80⁺ lung macrophages (Figure 7B). Injection of AP20187 into fibrotic MAFIA mice reduced the number of CD11c⁺CD11b^{var}F4/80⁺ macrophages ($P < 0.05$) (Figure 7B, *black bars*) but had no effect on the number of alveolar macrophages in BAL (Figure 7B, *red bars*).

Macrophage depletion in these mice reduced lung collagen ($P < 0.001$) (Figure 7C), restored compliance ($P < 0.01$) (Figure 7D), resulted in reduced BALF protein levels ($P < 0.01$) (Figure 7E), and led to an improvement in lung architecture (Figure 7F). Furthermore, macrophage depletion resulted in an almost complete loss of lung arginase activity, confirming that we had depleted alternatively programmed macrophages (Figure 7G). Akin to the lungs of TNF- α -treated fibrotic mice, 60% of the F4/80⁺ macrophages remaining in the lungs of AP20187-treated fibrotic MAFIA mice expressed an intermediate programming state reflected by the presence of arginase I and NOS2 (Figures 7H and 7I). Together, our findings show that the resolution of established fibrosis after conditional macrophage depletion reduces the number of alternatively programmed macrophages and phenocopies pulmonary delivery of TNF- α .

Discussion

Although much has been learned about the mechanisms underlying the development of pulmonary fibrosis, effective therapies to halt progression or reverse established disease are lacking. Indeed, conventional combination antiinflammatory/immunosuppressive therapy may be harmful (32). Here we show that local lung delivery of the proinflammatory cytokine TNF- α accelerates the resolution of established pulmonary fibrosis in mice. Furthermore, our results indicate that the antifibrotic effect of TNF- α is associated with reduced profibrotic alternative macrophage numbers and programming status. Taken together, these findings suggest that pulmonary delivery of TNF- α or the use of other strategies to reduce profibrotic lung macrophages may represent novel therapeutic approaches to ameliorate established pulmonary fibrosis.

Studies in bleomycin-instilled TNF- α receptor (TNF-R1/TNF-R2) double knockout mice and wild-type mice treated with anti-TNF- α antibodies initially suggested that TNF- α -receptor signaling is required for the development of pulmonary fibrosis (33, 34). However, fibrosis in the bleomycin model is now known to be dependent on the early induction of acute lung injury and inflammation, both of which are ablated in the absence of TNF-R1/TNF-R2

Figure 4. CD11c⁺CD11b^{var}F4/80⁺ macrophages are alternatively programmed during bleomycin-induced fibrosis. (A) Representative gating strategy to identify pulmonary macrophage subsets from whole lung digest in saline and bleomycin-instilled mice. Live cells were gated, followed by doublet exclusion on forward scatter (FSC) and side scatter (SSC). Macrophage subsets were identified as (blue circle, c) CD11c⁺CD11b^{var}F4/80⁺MHCII^{var} and (red circle, e) CD11c⁺CD11b⁺F4/80⁺Ly6G⁻. Dendritic cells (green square, d) were identified as SSC^{lo}CD11c⁺CD11b^{var}F4/80⁺MHCII^{hi} and neutrophils (blue circle, f) as CD11c⁺CD11b⁺F4/80⁺Ly6G⁺. The changes in mean fluorescence intensity (MFI) of CD206 and nitric oxide synthase 2 (NOS2) were determined compared with isotype controls. (B) Total macrophage subset numbers determined by flow cytometry from whole lung digest show a significant increase in CD11c⁺CD11b^{var}F4/80⁺ macrophages 3 weeks after bleomycin compared with 6 weeks and saline control mice (** $P < 0.01$). (C) Change in MFI of CD206 was significantly higher in CD11c⁺CD11b^{var}F4/80⁺ macrophages at 3 weeks after bleomycin compared with 6-week (* $P < 0.05$) and saline controls (*** $P < 0.001$). NOS2 expression did not change during the course of fibrosis. CD11c⁺CD11b⁺F4/80⁺ macrophages expressed very little CD206 or NOS2. (D) Immunofluorescent staining for F4/80 (red), arginase I (blue), and NOS2 (green) indicated that macrophages express arginase I but not NOS2 in fibrotic lungs obtained 3 weeks after bleomycin instillation. Macrophage-specific arginase I expression was decreased in lungs obtained 6 weeks after bleomycin instillation. (E) Quantification of fluorescence intensity by pixel counting revealed a significant increase in the percentage of arginase I-expressing macrophages (*** $P < 0.001$) and a significant decrease in the percentage of NOS2-expressing macrophages (** $P < 0.01$) 3 weeks after bleomycin instillation. (F) Arginase activity in whole lung homogenates was significantly increased at the peak of bleomycin-induced fibrosis (*** $P < 0.001$) but returned to baseline levels by 6 weeks.

Figure 5. Intratracheal delivery of TNF- α reduces CD11c⁺CD11b^{var}F4/80⁺ macrophage numbers and alternative programming. (A) Total macrophage numbers were significantly reduced in whole lung digests during the accelerated resolution initiated by TNF- α administration (** P < 0.01; *** P < 0.001). (B) CD206 expression was significantly reduced in CD11c⁺CD11b^{var}F4/80⁺ macrophages (** P < 0.01; *** P < 0.001), whereas NOS2 was significantly increased (** P < 0.01) in TNF- α -treated, bleomycin-instilled mice compared with saline-treated, bleomycin-instilled mice. (C) Immunofluorescent staining for F4/80 (red), arginase I (blue), and NOS2 (green) indicated that macrophages express NOS2 but not arginase I in the lungs of TNF- α -treated, bleomycin-instilled mice when compared with the exclusive arginase I expression in saline-treated, bleomycin-instilled mice. (D) Quantification of fluorescence intensity by pixel counting showed that there was a significant decrease in the percentage of arginase I-expressing macrophages (*** P < 0.001) and a significant increase in percentage of NOS2-expressing macrophages (*** P < 0.001) from TNF- α -treated, bleomycin-instilled mice compared with saline-treated, bleomycin-instilled mice. (E) Arginase activity was significantly reduced (* P < 0.05) in whole lung homogenates from TNF- α -treated, bleomycin-instilled mice compared with saline-treated, bleomycin-instilled mice.

receptor signaling (33). It is also known that TNF-R1 and TNF-R2 ligate and signal in response to TNF- α and lymphotoxin- α (LT- α) (35) and that combined deficiency or neutralization of TNF- α and LT- α results in resistance to bleomycin-induced acute lung injury and fibrosis (36, 37). In confirmation of a previous study (29), we observed robust inflammation and injury in the lungs of bleomycin-instilled TNF- α -deficient mice, suggesting that TNF- α and LT- α exhibit redundant roles in the initiation of acute lung injury and inflammation. However, our finding that fibrosis and alternative macrophage programming were prolonged in TNF- α ^{-/-} mice indicates that TNF- α alone

exerts a nonredundant and previously unknown role in the resolution of established fibrosis. This conclusion is also compatible with previous studies in which lung-specific expression of TNF- α by intratracheal delivery of TNF- α -expressing adenoviruses to rats or by transgenic expression in alveolar type II cells in mice promoted robust lymphocytic infiltration and emphysematous changes in the lung parenchyma but minimal fibrosis (38, 39). In agreement with earlier studies (40), measurements of lung TNF- α levels in wild-type mice indicated that TNF- α was only detectable during the first week after bleomycin instillation. This finding suggests that the antifibrotic effects of endogenously

produced TNF- α are initiated during the early injury/inflammatory phase even though they are not manifested until several weeks later. Similarly, early production of IFN- γ in bleomycin-instilled mice is also thought to promote late-phase antifibrotic effects (41). Thus, our findings establish for the first time, to our knowledge, that TNF- α is antifibrotic in the setting of established fibrosis.

The role of macrophages in the development of pulmonary fibrosis has been under intense study for the past three decades. Using accepted programming markers, we found that the majority of CD11c⁺CD11b^{var}F4/80⁺ macrophages in fibrotic lung tissues were alternatively

Figure 6. Macrophages maintain alternative programming marker expression during the impaired resolution of fibrosis seen in TNF- $\alpha^{-/-}$ mice. (A) Immunofluorescent staining for F4/80 (red), arginase I (blue), and NOS2 (green) indicates that macrophages continue to express arginase I and not NOS2 in fibrotic lungs obtained for up to 8 weeks after bleomycin instillation compared with wild-type mice that lose arginase I staining during the spontaneous resolution seen at 6 to 8 weeks after bleomycin administration. (B) Quantification of fluorescence intensity by pixel density indicated that there was no significant change in the percentage of arginase I expressing macrophages in macrophages from TNF- $\alpha^{-/-}$ mice for up to 8 weeks after bleomycin instillation ($^{***}P < 0.001$). (C) Compared with wild-type mice, arginase activity is significantly elevated ($^{*}P < 0.05$) in whole lung homogenates from TNF- $\alpha^{-/-}$ mice for up to 8 weeks after bleomycin instillation.

programmed, consistent with earlier reports (8, 42, 43). Herein, we established that the mechanism by which TNF- α resolves established pulmonary fibrosis involves two components. First, pulmonary delivery of TNF- α promotes a direct down-regulation in the alternative programming status of CD11c⁺CD11b^{var}F4/80⁺ macrophages and an increase in their expression of the classical programming marker NOS2. The function of these dual-positive macrophages remains to be determined. Perhaps they represent a transitional programming state as the profibrotic macrophages are reprogrammed to a gene expression signature more akin to classically activated macrophages. Alternatively, the dual-positive macrophages may represent a distinct and potentially antifibrotic programming state, similar to the so-called

“restorative” macrophages observed in mice during the spontaneous resolution of hepatic fibrosis (20). Second, TNF- α instillation into fibrotic mice reduced the number of CD11c⁺CD11b^{var}F4/80⁺ macrophages. The mechanisms underlying this reduction in macrophage number is unknown but could include TNF- α -induced CCR2 down-regulation leading to reduced monocyte influx (44) and/or increased susceptibility of this subset to TNF- α induced apoptosis (45, 46). Conditional depletion of CD11c⁺CD11b^{var}F4/80⁺ and CD11c⁻CD11b⁺F4/80⁺ macrophages in fibrotic MAFIA mice phenocopied the effects of therapeutically delivered TNF- α in resolving established fibrosis, suggesting that the loss of alternatively programmed CD11c⁺CD11b^{var}F4/80⁺ macrophages is responsible for the accelerated resolution

of fibrosis in MAFIA mice. The dimerizing drug AP20187 did not deplete alveolar macrophages, possibly because it did not efficiently permeate the alveolar-capillary barrier. Moreover, as was seen in TNF- α -instilled fibrotic mice, the alveolar macrophages that persisted in AP20187-treated MAFIA mice expressed arginase I and NOS2, also raising the question of whether these cells represent so-called “restorative” macrophages (20).

Although our findings suggest that the antifibrotic action of TNF- α is mediated by reducing the number and alternative programming status of lung macrophages, the resolution of established fibrosis is a complex process that also requires (1) the elimination of collagen-producing fibroblasts and myofibroblasts, (2) degradation and phagocytosis of

Figure 7. Macrophage depletion in MAFIA (Macrophage Fas-Induced Appoptosis) mice results in decreased fibrosis. (A) Schematic showing the AP20187 treatment regimen that was used to deplete macrophages after bleomycin instillation in MAFIA mice. (B) Total macrophage numbers determined by flow cytometry from whole lung digest from bleomycin-instilled mice showed a significant decrease in the CD11c⁺CD11b^{var}F4/80⁺ and CD11c⁺CD11b⁺F4/80⁺ subsets after delivery of AP20187 ($P < 0.05$). There was no significant depletion of alveolar macrophages ($P = 0.868$). (C) Hydroxyproline levels were significantly reduced in bleomycin-instilled mice after delivery of AP20187 ($***P < 0.001$) compared with mice receiving bleomycin + vehicle. (D) Static compliance was significantly improved in AP20187-treated bleomycin-instilled MAFIA mice when compared with bleomycin-instilled mice receiving vehicle alone ($**P < 0.01$). (E) Total BALF protein levels were significantly reduced ($**P < 0.01$) in AP20187-treated

deposited collagen, and (3) reepithelialization of the denuded and damaged basement membranes. We have previously shown that TNF- α contributes to the elimination of fibroblasts and myofibroblasts via sensitization to Fas-induced apoptosis (47, 48). TNF- α also inhibits fibroblast proliferation and TGF- β 1-induced fibroblast-myofibroblast transdifferentiation and matrix contraction (49). In addition, TNF- α induces the expression of metalloproteinases 2 and 9 (50, 51), which may contribute to the elimination of collagen. Alveolar macrophage-derived TNF- α augments alveolar epithelial cell proliferation and reepithelialization (52), consistent with our suggestion that the remaining alveolar macrophages in the lungs of TNF- α - and macrophage-depleted fibrotic mice may contribute to the restoration of normal lung structure and function. Future studies will address these issues.

Our findings also raise questions about possible relationships between the inflammatory potential of TNF- α and its antifibrotic activity in the setting

of established fibrosis. The role of inflammation in the development of pulmonary fibrosis continues to be debated. Persistent inflammation of the lung parenchyma undoubtedly leads to epithelial cell injury and damage to the alveolar-capillary basement membranes, in turn initiating a fibrotic response (53). However, fibrosis has also been proposed to develop in the absence of a robust inflammatory response via chronic alveolar epithelial cell stress, injury, and apoptosis (54). Given that TNF- α is a proinflammatory cytokine, we speculate that a mild inflammatory response, initiated by TNF- α , may be beneficial in resolving established fibrosis. This view is supported by earlier studies showing that bleomycin-induced pulmonary fibrosis in rats is worsened by neutrophil depletion (55). Mild neutrophilic inflammation has also been shown to augment airway reepithelialization in ozone-exposed monkeys (56). Indeed, it is tempting to speculate that the increased mortality and respiratory failure seen in patients with IPF receiving combined

antiinflammatory and immunosuppressive combination therapy (azathioprine, prednisolone, and N-acetylcysteine) in the recently terminated PANTHER-IPF trial may be related to a reduction in “beneficial inflammation” (32). Our findings also raise questions about the potential detrimental effects of anti-TNF- α therapy in patients with interstitial lung disease (57, 58). In contrast, a clear implication of the current findings is that controlled pulmonary delivery of TNF- α or activation of its downstream receptor signaling pathways may represent a novel therapeutic approach to reduce the fibrotic burden in patients with pulmonary fibrosis without the adverse effects associated with systemic TNF- α delivery. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Ben Edelman and Linda Remigio for technical assistance.

References

1. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am J Respir Crit Care Med* 2000;161:646–664.
2. Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2006;174:810–816.
3. Myers JL, Katzenstein AL. Epithelial necrosis and alveolar collapse in the pathogenesis of usual interstitial pneumonia. *Chest* 1988;94:1309–1311.
4. Thannickal VJ, Horowitz JC. Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* 2006;3:350–356.
5. Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991;88:6642–6646.
6. White ES, Mantovani AR. Inflammation, wound repair, and fibrosis: reassessing the spectrum of tissue injury and resolution. *J Pathol* 2013;229:141–144.
7. Kuhn C III, Boldt J, King TE Jr, Crouch E, Vartio T, McDonald JA. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am Rev Respir Dis* 1989;140:1693–1703.
8. Gibbons MA, MacKinnon AC, Ramachandran P, Dhaliwal K, Duffin R, Phytian-Adams AT, van Rooijen N, Haslett C, Howie SE, Simpson AJ, et al. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am J Respir Crit Care Med* 2011;184:569–581.
9. Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* 2010;30:245–257.
10. Fernandez IE, Eickelberg O. New cellular and molecular mechanisms of lung injury and fibrosis in idiopathic pulmonary fibrosis. *Lancet* 2012;380:680–688.
11. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3:23–35.
12. Riches DW. Signalling heterogeneity as a contributing factor in macrophage functional diversity. *Semin Cell Biol* 1995;6:377–384.
13. Wynne MW, Riches DW. Induction of macrophage insulin-like growth factor-I expression by the Th2 cytokines IL-4 and IL-13. *J Immunol* 2003;171:3550–3559.

Figure 7. (Continued). bleomycin-instilled MAFIA mice compared with vehicle-treated bleomycin-instilled MAFIA mice. (F) Reduced lung collagen in Masson’s trichrome-stained lung sections from AP20187-treated bleomycin-instilled MAFIA mice compared with vehicle-treated bleomycin-instilled MAFIA mice. Original magnification: $\times 20$. (G) Arginase activity in whole lung homogenates was significantly reduced ($*P < 0.05$) in AP20187-treated bleomycin-instilled MAFIA mice compared with vehicle-treated bleomycin-instilled MAFIA mice. (H) Immunofluorescent staining for F4/80 (red), arginase I (blue), and NOS2 (green) revealed that the remaining alveolar macrophages in AP20187-treated, bleomycin-instilled MAFIA mice exhibit a unique intermediate programming state in which both markers were expressed. (I) Quantitative fluorescence analysis by pixel counting showed that there was no significant difference ($P = 0.7408$) in the percentage of arginase I-positive macrophages between AP20187 and vehicle-treated, bleomycin-instilled MAFIA mice. There was a significant increase in the percentage of NOS2-staining macrophages ($***P < 0.001$).

14. Khalil N, Berezney O, Sporn M, Greenberg AH. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med* 1989;170:727-737.
15. Krishna G, Liu K, Shigemitsu H, Gao M, Raffin TA, Rosen GD. PG490-88, a derivative of triptolide, blocks bleomycin-induced lung fibrosis. *Am J Pathol* 2001;158:997-1004.
16. Piguet PF, Collart MA, Grau GE, Sappino AP, Vassalli P. Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* 1990;344:245-247.
17. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 2005;175:342-349.
18. Lake FR, Noble PW, Henson PM, Riches DW. Functional switching of macrophage responses to tumor necrosis factor- α (TNF α) by interferons: implications for the pleiotropic activities of TNF α . *J Clin Invest* 1994;93:1661-1669.
19. Laszlo DJ, Henson PM, Remigio LK, Weinstein L, Sable C, Noble PW, Riches DW. Development of functional diversity in mouse macrophages: mutual exclusion of two phenotypic states. *Am J Pathol* 1993;143:587-597.
20. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, Hartland SN, Snowden VK, Cappon A, Gordon-Walker TT, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA* 2012;109:E3186-E3195.
21. Redente EF, Cosgrove GP, Keith RC, Sergew A, Riches DWH. Tnf- α facilitates pulmonary fibrosis resolution through alternative programming of the lung microenvironment [abstract]. *Am J Respir Crit Care Med Abstract ATS #506* 2010.
22. Redente EF, Jacobsen KM, Solomon JJ, Lara AR, Faubel S, Keith RC, Henson PM, Downey GP, Riches DW. Age and sex dimorphisms contribute to the severity of bleomycin-induced lung injury and fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L510-L518.
23. Burnett SH, Kersten EJ, Zhang J, Zeng L, Straley SC, Kaplan AM, Cohen DA. Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *J Leukoc Biol* 2004;75:612-623.
24. Keith RC, Powers JL, Redente EF, Sergew A, Martin RJ, Gizinski A, Holers VM, Sakaguchi S, Riches DW. A novel model of rheumatoid arthritis-associated interstitial lung disease in SKG mice. *Exp Lung Res* 2012;38:55-66.
25. Foster B, Calman P, Fei L, Whitmire J, Whitton J. Detection of intracellular cytokines by flow cytometry: current protocols in immunology. New York: John Wiley & Sons, Inc.; 2007. p. 6.24.21-26.24.21.
26. Redente EF, Higgins DM, Dwyer-Nield LD, Orme IM, Gonzalez-Juarrero M, Malkinson AM. Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation. *J Leukoc Biol* 2010;88:159-168.
27. Takemoto K, Ogino K, Shibamori M, Gondo T, Hitomi Y, Takigawa T, Wang DH, Takaki J, Ichimura H, Fujikura Y, et al. Transiently, paralleled upregulation of arginase and nitric oxide synthase and the effect of both enzymes on the pathology of asthma. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1419-L1426.
28. Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L152-L160.
29. Kuroki M, Noguchi Y, Shimono M, Tomono K, Tashiro T, Obata Y, Nakayama E, Kohno S. Repression of bleomycin-induced pneumopathy by TNF. *J Immunol* 2003;170:567-574.
30. Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada Calvo F, Henry E, Closset R, Dewals B, Thielen C, Gustin P, et al. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest* 2009;119:3723-3738.
31. Lagranderie M, Nahori MA, Balazuc AM, Kiefer-Biasizzo H, Lapa e Silva JR, Milon G, Marchal G, Vargaftig BB. Dendritic cells recruited to the lung shortly after intranasal delivery of *Mycobacterium bovis* BCG drive the primary immune response towards a type 1 cytokine production. *Immunology* 2003;108:352-364.
32. Raghu G, Anstrom KJ, King TE Jr, Lasky JA, Martinez FJ; Idiopathic Pulmonary Fibrosis Clinical Research Network. Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. *N Engl J Med* 2012;366:1968-1977.
33. Ortiz LA, Lasky J, Hamilton RF Jr, Holian A, Hoyle GW, Banks W, Peschon JJ, Brody AR, Lungarella G, Friedman M. Expression of TNF and the necessity of TNF receptors in bleomycin-induced lung injury in mice. *Exp Lung Res* 1998;24:721-743.
34. Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med* 1989;170:655-663.
35. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003;3:745-756.
36. Piguet PF, Kaufman S, Barazzzone C, Muller M, Ryffel B, Eugster HP. Resistance of TNF/LT α double deficient mice to bleomycin-induced fibrosis. *Int J Exp Pathol* 1997;78:43-48.
37. Piguet PF, Vesin C. Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice. *Eur Respir J* 1994;7:515-518.
38. Sime PJ, Marr RA, Gaudie D, Xing Z, Hewlett BR, Graham FL, Gaudie J. Transfer of tumor necrosis factor- α to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with induction of transforming growth factor- β 1 and myofibroblasts. *Am J Pathol* 1998;153:825-832.
39. Fujita M, Shannon JM, Morikawa O, Gaudie J, Hara N, Mason RJ. Overexpression of tumor necrosis factor- α diminishes pulmonary fibrosis induced by bleomycin or transforming growth factor- β . *Am J Respir Cell Mol Biol* 2003;29:669-676.
40. Phan SH, Kunkel SL. Lung cytokine production in bleomycin-induced pulmonary fibrosis. *Exp Lung Res* 1992;18:29-43.
41. Jiang D, Liang J, Hodge J, Lu B, Zhu Z, Yu S, Fan J, Gao Y, Yin Z, Homer R, et al. Regulation of pulmonary fibrosis by chemokine receptor CXCR3. *J Clin Invest* 2004;114:291-299.
42. Prasse A, Pechkovsky DV, Toews GB, Jungraithmayr W, Kollert F, Goldmann T, Vollmer E, Müller-Quernheim J, Zissel G. A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18. *Am J Respir Crit Care Med* 2006;173:781-792.
43. Zhang H, Han G, Liu H, Chen J, Ji X, Zhou F, Zhou Y, Xie C. The development of classically and alternatively activated macrophages has different effects on the varied stages of radiation-induced pulmonary injury in mice. *J Radiat Res (Tokyo)* 2011;52:717-726.
44. Weber C, Draude G, Weber KS, Wübert J, Lorenz RL, Weber PC. Downregulation by tumor necrosis factor- α of monocyte CCR2 expression and monocyte chemotactic protein-1-induced transendothelial migration is antagonized by oxidized low-density lipoprotein: a potential mechanism of monocyte retention in atherosclerotic lesions. *Atherosclerosis* 1999;145:115-123.
45. Dreschers S, Gille C, Haas M, Grosse-Ophoff J, Schneider M, Leibler A, Bühring HJ, Orlikowsky TW. Infection-induced bystander-apoptosis of monocytes is TNF- α -mediated. *PLoS ONE* 2013;8:e53589.
46. Xaus J, Comalada M, Valledor AF, Lloberas J, López-Soriano F, Argilés JM, Bogdan C, Celada A. LPS induces apoptosis in macrophages mostly through the autocrine production of TNF- α . *Blood* 2000;95:3823-3831.
47. Wynes MW, Edelman BL, Kostyk AG, Edwards MG, Coldren C, Groshong SD, Cosgrove GP, Redente EF, Bamberg A, Brown KK, et al. Increased cell surface Fas expression is necessary and sufficient to sensitize lung fibroblasts to Fas ligation-induced apoptosis: implications for fibroblast accumulation in idiopathic pulmonary fibrosis. *J Immunol* 2011;187:527-537.
48. Frankel SK, Cosgrove GP, Cha SI, Cool CD, Wynes MW, Edelman BL, Brown KK, Riches DW. TNF- α sensitizes normal and fibrotic human lung fibroblasts to Fas-induced apoptosis. *Am J Respir Cell Mol Biol* 2006;34:293-304.
49. Goldberg MT, Han YP, Yan C, Shaw MC, Garner WL. TNF- α suppresses α -smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound healing. *J Invest Dermatol* 2007;127:2645-2655.
50. Sarén P, Welgus HG, Kovanen PT. TNF- α and IL-1 β selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol* 1996;157:4159-4165.

51. Zhang HS, Wang SQ. Salvianolic acid B from *Salvia miltiorrhiza* inhibits tumor necrosis factor- α (TNF- α)-induced MMP-2 upregulation in human aortic smooth muscle cells via suppression of NAD(P)H oxidase-derived reactive oxygen species. *J Mol Cell Cardiol* 2006;41:138–148.
52. Cakarova L, Marsh LM, Wilhelm J, Mayer K, Grimminger F, Seeger W, Lohmeyer J, Herold S. Macrophage tumor necrosis factor- α induces epithelial expression of granulocyte-macrophage colony-stimulating factor: impact on alveolar epithelial repair. *Am J Respir Crit Care Med* 2009;180:521–532.
53. Khalil N, O'Connor RN, Unruh HW, Warren PW, Flanders KC, Kemp A, Berezney OH, Greenberg AH. Increased production and immunohistochemical localization of transforming growth factor- β in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 1991;5:155–162.
54. King TE Jr, Pardo A, Selman M. Idiopathic pulmonary fibrosis. *Lancet* 2011;378:1949–1961.
55. Thrall RS, Phan SH, McCormick JR, Ward PA. The development of bleomycin-induced pulmonary fibrosis in neutrophil-depleted and complement-depleted rats. *Am J Pathol* 1981;105:76–81.
56. Hyde DM, Miller LA, McDonald RJ, Stovall MY, Wong V, Pinkerton KE, Wegner CD, Rothlein R, Plopper CG. Neutrophils enhance clearance of necrotic epithelial cells in ozone-induced lung injury in rhesus monkeys. *Am J Physiol* 1999;277:L1190–L1198.
57. Ostor AJ, Crisp AJ, Somerville MF, Scott DG. Fatal exacerbation of rheumatoid arthritis associated fibrosing alveolitis in patients given infliximab. *BMJ* 2004;329:1266.
58. Chatterjee S. Severe interstitial pneumonitis associated with infliximab therapy. *Scand J Rheumatol* 2004;33:276–277.