

# Integrating mechanisms of pulmonary fibrosis

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**Pulmonary fibrosis is a highly heterogeneous and lethal pathological process with limited therapeutic options. Although research on the pathogenesis of pulmonary fibrosis has frequently focused on the mechanisms that regulate the proliferation, activation, and differentiation of collagen-secreting myofibroblasts, recent studies have identified new pathogenic mechanisms that are critically involved in the initiation and progression of fibrosis in a variety of settings. A more detailed and integrated understanding of the cellular and molecular mechanisms of pulmonary fibrosis could help pave the way for effective therapeutics for this devastating and complex disease.**

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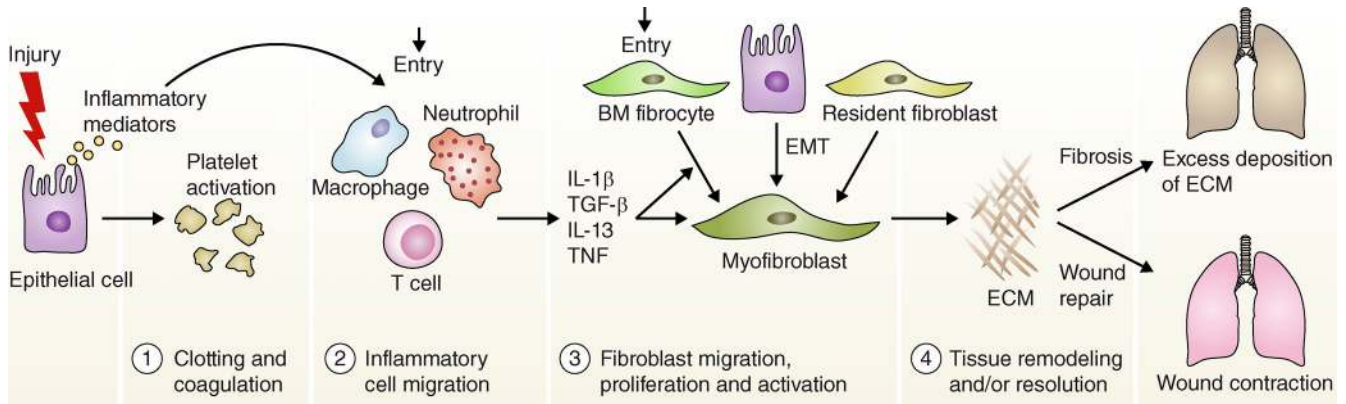
Abbreviations used: BAL, bronchoalveolar lavage; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; IPF, idiopathic pulmonary fibrosis; miRNA, micro RNA; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homologue; ROS, reactive oxygen species; TSLP, thymic stromal lymphopoietin; WISP-1, WNT1-inducible signaling protein-1.

Pulmonary fibrosis is a lung disease that is refractory to treatment and carries a high mortality rate. It includes a heterogeneous group of lung disorders characterized by the progressive and irreversible destruction of lung architecture caused by scar formation that ultimately leads to organ malfunction, disruption of gas exchange, and death from respiratory failure. Idiopathic pulmonary fibrosis (IPF), a particularly severe form of pulmonary fibrosis with unknown etiology has a life expectancy of 2–6 yr after diagnosis (Selman et al., 2001). Lung fibrosis can also develop after viral infections and after exposure to radiotherapy, chemotherapeutic drugs, and aerosolized environmental toxins (Denham and Hauer-Jensen, 2002; Kelly et al., 2002; Fubini and Hubbard, 2003; Chen and Stubbe, 2005). It also occurs in some bone marrow transplant recipients suffering from chronic graft versus host disease and in a subset of individuals with chronic inflammatory diseases like scleroderma and rheumatoid arthritis (Wolff et al., 2002; Young et al., 2007). Currently, the only effective treatment available for progressive lung fibrosis is lung transplantation.

Repair of damaged tissues is a fundamental biological mechanism that allows the ordered replacement of dead or damaged cells after injury, a process critically important for survival (Wynn, 2007). However, if this process becomes dysregulated, it can lead to the development of a permanent fibrotic “scar,” which is characterized by the excess accumulation of extracellular matrix (ECM) components (e.g., hyaluronic

acid, fibronectin, proteoglycans, and interstitial collagens) at the site of tissue injury. Consequently, fibrogenesis is often defined as an out of control wound healing response. Wound repair has four distinct stages that include a clotting/coagulation phase, an inflammatory phase, a fibroblast migration/proliferation phase, and a final remodeling phase where normal tissue architecture is restored (Fig. 1). In the earliest stages after tissue damage, epithelial cells and/or endothelial cells release inflammatory mediators that initiate an antifibrinolytic-coagulation cascade that triggers clotting and development of a provisional ECM. Platelet aggregation and subsequent degranulation in turn promotes blood vessel dilation and increased permeability, allowing efficient recruitment of inflammatory cells (e.g., neutrophils, macrophages, lymphocytes, and eosinophils) to the site of injury. Neutrophils are the most abundant inflammatory cell at the earliest stages of wound healing, but are quickly replaced by macrophages after neutrophil degranulation. During this initial leukocyte migration phase, activated macrophages and neutrophils debride the wound and eliminate any invading organisms. They also produce a variety of cytokines and chemokines that amplify the inflammatory response and trigger fibroblast proliferation

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**Figure 1. Disruptions in normal wound healing contribute to the development of pulmonary fibrosis.** Wound healing has four distinct stages: a clotting/coagulation phase (1), an inflammatory cell migration phase (2), a fibroblast migration/proliferation/activation phase (3), and a tissue remodeling and resolution phase (4). After lung injury, epithelial cells release inflammatory mediators that initiate an antifibrinolytic coagulation cascade, which triggers platelet activation and blood clot formation. This is followed by entry of leukocytes (e.g., neutrophils, macrophages, and T cells). The recruited leukocytes secrete profibrotic cytokines such as IL-1 $\beta$ , TNF, IL-13, and TGF- $\beta$ . The activated macrophages and neutrophils also remove dead cells and eliminate any invading organisms. In the subsequent phase, fibrocytes from the bone marrow and resident fibroblasts proliferate and differentiate into myfibroblasts, which release ECM components. Fibroblasts and myfibroblasts may also be derived from epithelial cells undergoing EMT. In the final remodeling and resolution phase, activated myfibroblasts can promote wound repair, leading to wound contraction and restoration of blood vessels. However, fibrosis often develops if any stage in the tissue repair program is dysregulated or when the lung-damaging stimulus persists.

and recruitment. Myfibroblasts are recruited from a variety of sources including local mesenchymal cells, bone marrow progenitors (called fibrocytes), and via a process called epithelial–mesenchymal transition (EMT), wherein epithelial cells transdifferentiate into fibroblast-like cells. The overall importance of each fibroblast population, however, remains unclear. Once fibroblasts become activated, they transform into  $\alpha$ -smooth muscle actin–expressing myfibroblasts that secrete ECM components. Finally, in the wound maturation/remodeling phase, myfibroblasts promote wound contraction, a process where the edges of the wound migrate toward the center and epithelial/endothelial cells divide and migrate over the temporary matrix to regenerate the damaged tissue. Fibrosis develops when the wound is severe, the tissue-damaging irritant persists, or when the repair process becomes dysregulated. Thus, many stages in the wound repair process can go awry and contribute to scar formation, likely explaining the complex nature of pulmonary fibrosis.

Although the relative importance of inflammation in the progression of pulmonary fibrosis has been debated, many forms of the disease are believed to be induced, at least initially, by a strong inflammatory response (Crystal et al., 2002). Although some types of pulmonary fibrosis maintain a significant inflammatory component throughout the course of the disease, other forms like IPF are often characterized as exhibiting highly progressive fibrotic disease in the absence of detectable inflammation (Thannickal et al., 2004). In this case, it has been hypothesized that intrinsic defects in the wound healing response involving lung epithelial cells and fibroblasts contribute to the progression of fibrosis. The fact that an active inflammatory response is not a strict prerequisite likely explains why standard antiinflammatory therapies, including corticosteroids and cytotoxic agents have shown little efficacy

in IPF (Demedts et al., 2005). An improved clinical, radiographical, and histopathologic classification of the various forms and stages of pulmonary fibrosis and a more detailed understanding of the molecular mechanisms of fibrogenesis is needed so that therapies can be better tailored to specifically attack the underlying causes of the disease.

Although many forms of pulmonary fibrosis can be effectively modeled and studied in rodents including drug (e.g., bleomycin), particulate-matter (e.g., asbestos and silica), radiation, bronchiolitis obliterans, and chronic graft-versus-host-induced pulmonary fibrosis, it remains unclear whether any of the experimental models truly duplicate the idiopathic form of the disease that is commonly seen in humans (Moore and Hogaboam, 2008). Nevertheless, many important advances have been generated from rodent models, which have been dominated by transgenic and knockout mice that display either enhanced or decreased susceptibility to pulmonary fibrosis. These important studies have greatly expanded our understanding of the mechanisms of pulmonary fibrosis and are a major focus of this review.

Whereas nearly two decades of research have suggested that TGF- $\beta$  plays a central role in the pathogenesis of pulmonary fibrosis by promoting the activation, proliferation, and differentiation of epithelial cells and collagen-producing myfibroblasts (Border and Noble, 1994), little progress has been made in moving TGF- $\beta$  pathway inhibitors from the bench to the bedside (Kisseleva and Brenner, 2008). In fact, there are still currently no approved drugs that specifically target any proposed mechanism of pulmonary fibrosis. Over the past few years several novel antifibrotic strategies have been described that do not involve targeting the TGF- $\beta$  signaling pathway directly (Fig. 2). This review will highlight some of these exciting discoveries and illustrate how these

unique targets and approaches might be exploited to treat this highly heterogeneous and complex disease. It also emphasizes that although many important advances have been made over the past few years, a great deal of work is needed before we can fully integrate all of the pathways and mechanisms that regulate the pathogenesis of pulmonary fibrosis.

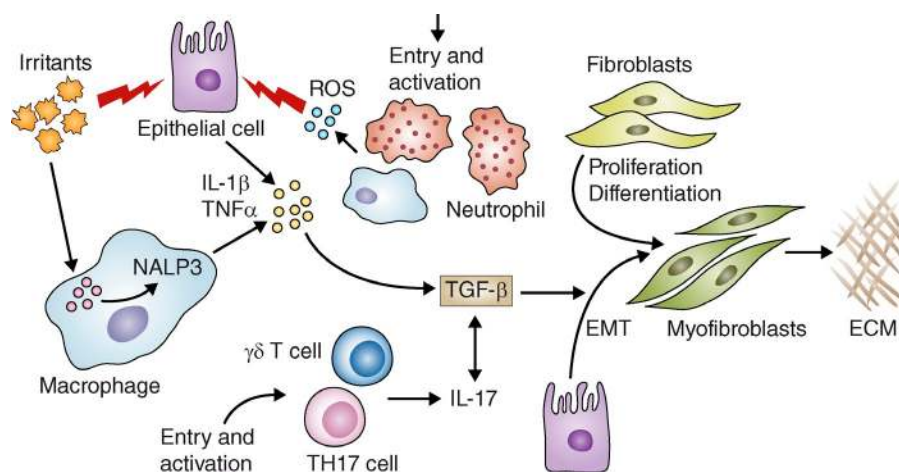
### A role for proinflammatory mediators in pulmonary fibrosis

It seems clear that inflammatory mediators play a role in both the initiation and progression of some forms of pulmonary fibrosis (Bringardner et al., 2008). Surgical biopsies and serum samples from patients with idiopathic or systemic sclerosis-associated pulmonary fibrosis display elevated levels of TNF and mice that overexpress the cytokine in the lung develop progressive pulmonary fibrosis (Piguet et al., 1993; Miyazaki et al., 1995; Hasegawa et al., 1997). Macrophages and many other cell types produce TNF in the lung after exposure to silica, asbestos, and bleomycin (Piguet et al., 1990; Piguet and Vesin, 1994; Zhang et al., 1993). Clinical trials were recently initiated to investigate if TNF pathway inhibitors like Etanercept might be beneficial in the treatment of IPF (Raghu et al., 2008). However, although inhibitors of TNF have shown efficacy in some pulmonary fibrosis models, because TNF can inhibit collagen synthesis in myofibroblasts, TNF antagonists might have the undesired effect of worsening the disease (Sivik et al., 2000). Indeed, TNF blockade exacerbated pulmonary fibrosis in rheumatoid arthritis patients taking methotrexate (Thavarajah et al., 2009). Therefore, it remains unclear if TNF blockade will ultimately prove beneficial in IPF or other forms of pulmonary fibrosis.

Like TNF, IL-1 $\beta$  can induce acute lung injury and may contribute to the progression of pulmonary fibrosis (Kolb et al., 2001). Interestingly, IL-1 $\beta$ -induced fibrosis is associated with increased expression of TNF, suggesting that IL-1 $\beta$ - and TNF-triggered fibrosis might be linked mechanistically. The neutrophil attracting CXC chemokines CXCL1 (KC) and CXCL2 (MIP-2) are also increased by IL-1 $\beta$ , as are the profibrotic cytokines platelet-derived growth factor (PDGF) and TGF- $\beta$ 1, illustrating how acute lung injury initiated by

proinflammatory cytokines and neutrophils can quickly evolve to a progressive fibrotic response (Kolb et al., 2001; Lappalainen et al., 2005). The IL-1 receptor antagonist dampens the profibrotic effects of IL-1 $\beta$  (Ortiz et al., 2007), and studies with IL-1R1 $^{-/-}$ , MyD88 $^{-/-}$ , ASC $^{-/-}$ , Caspase-1 $^{-/-}$ , and Nalp3 $^{-/-}$  mice have suggested that uric acid (induced by bleomycin), asbestos, and silica are detected by the Nalp3 inflammasome in macrophages, leading to IL-1R1/MyD88 signaling that is critical in the pathogenesis of particulate matter and bleomycin-driven fibrosis (Gasse et al., 2007, 2009; Cassel et al., 2008; Dostert et al., 2008).

IL-17A has also been implicated in the pathogenesis of pulmonary fibrosis (Langrish et al., 2005; Bettelli et al., 2006; Simonian et al., 2009; Wilson et al., 2010). IL-17A is increased in the bronchoalveolar lavage (BAL) fluid of patients with IPF (Wilson et al., 2010). IL-17A expression is associated with the persistent neutrophilia observed in a variety of lung disorders, including bacterial pneumonia and cystic fibrosis (Ye et al., 2001; Decraene et al., 2010; Brodlie et al., 2011; Hsu et al., 2011). Notably, recruitment of neutrophils to the BAL is an important predictor of early mortality in IPF patients (Kinder et al., 2008). IL-17A- and IL-17RA-dependent signaling are also important for the development of pulmonary fibrosis after exposure to bleomycin or *Saccharopolyspora rectivirgula*, a bacterium that causes hypersensitivity pneumonitis in rodent models (Simonian et al., 2009; Wilson et al., 2010). Detailed mechanistic studies in mice with bleomycin-induced fibrosis suggested that IL-23 and possibly IL-12 are important inducers of IL-17A-dependent fibrosis. Although  $\gamma\delta$  T cells are an important source of IL-17A, CD4 $^{+}$  T cells were identified as the dominant producer of IL-17A after bleomycin exposure. Interestingly, IL-22 produced by  $\gamma\delta$  T cells protected mouse lungs from *Bacillus subtilis*-induced fibrosis, perhaps suggesting opposing roles for CD4 $^{+}$  Th17 cells and IL-22-expressing  $\gamma\delta$  T cells in the development of pulmonary fibrosis (Simonian et al., 2010). Bleomycin-induced IL-17A production is also highly



**Figure 2. Proinflammatory and profibrotic mediators in the initiation and maintenance of fibrosis.** Irritants like silica, asbestos, and bleomycin (uric acid) can injure lung epithelial cells and can be detected by the Nalp3 inflammasome in macrophages. These irritants stimulate the production of ROS, chemokines, and cytokines. These inflammatory mediators enhance the recruitment and activation of leukocytes at the site of tissue injury. For example, IL-1 $\beta$  induces the activation of ROS-expressing neutrophils, which can further damage epithelial cells. IL-1 $\beta$  also promotes production of TGF- $\beta$ 1, an important profibrotic cytokine that triggers fibroblast proliferation and activation. TGF- $\beta$  also targets epithelial cells, inducing EMT and the formation of ECM-producing myofibroblasts. TGF- $\beta$ 1 further exacerbates the inflammatory response by stimulating the differentiation of Th17 cells. [Interactive PPT slides for this figure are available online.](#)



dependent on TGF- $\beta$ 1 signaling, and recombinant IL-17A-driven fibrosis is dependent on the downstream profibrotic activity of TGF- $\beta$ 1, suggesting codependent roles for IL-17A and TGF- $\beta$ 1 in the development of pulmonary fibrosis (Wilson et al., 2010).

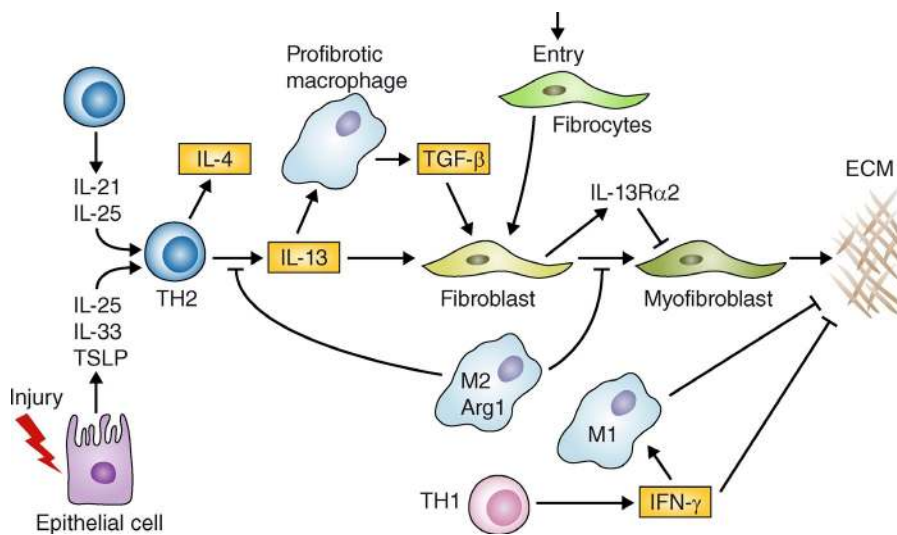
Oxidative stress also perpetuates profibrotic inflammatory responses. Indeed, activation of the Nalp3 inflammasome and IL-1 $\beta$  secretion are largely driven by reactive oxygen species (ROS) derived by ROS-generating mitochondria (Naik and Dixit, 2011; Zhou et al., 2011) and NADPH oxidase (NOX) family members expressed in macrophages and neutrophils (Cassel et al., 2008; Dostert et al., 2008). NOX4 activity is induced in the lungs of mice after particle phagocytosis and promotes fibrogenesis in two distinct models of lung injury (Hecker et al., 2009). NOX4 is also increased in cases of human IPF. Mechanistically, NOX4 is required for TGF- $\beta$ 1-induced myofibroblast differentiation, ECM synthesis, and fibroblast contractility.

In addition to directly promoting fibroblast activation, ROS, IL-1 $\beta$ , and TNF can also promote fibrosis by increasing expression of plasminogen activator inhibitor 1, which functions as a physiological inhibitor of the ECM degrading plasmin/plasminogen activator system that protects mouse lungs from fibrosis (Liu, 2008). The plasmin/plasminogen activation system also increases production of prostaglandin E2 and COX-2, which exhibit potent antifibrotic activity in the lung (Park and Christman, 2006; Bauman et al., 2010). The antifibrotic mediator prostaglandin E2 is also induced in the lungs of mice deficient in cytosolic phospholipase A(2), suggesting that antagonists of cytosolic phospholipase A(2) might represent an additional strategy to treat fibrotic lung disease (Nagase et al., 2002; Peters-Golden et al., 2002; Wilborn et al., 1996). It is important to note, however, that not all prostaglandins exhibit antifibrotic

activity, as prostaglandin F-2  $\alpha$ , found in the BAL fluid of subjects with IPF, was shown to stimulate collagen production in fibroblasts through a TGF- $\beta$ 1-independent/PG(F) receptor-dependent mechanism (Oga et al., 2009). Nevertheless, these observations illustrate an important role for various mediators in inflammation-driven pulmonary fibrosis (Fig. 2).

### Regulation by Th2 responses and IL-13

There is a great deal of evidence that CD4<sup>+</sup> Th1 and Th2 cells play important roles during the inflammatory/maintenance phase of pulmonary fibrosis (Wynn, 2004). Indeed, cytokines associated with CD4<sup>+</sup> Th1 and Th2 cells have exhibited contrasting activity in fibrogenesis (Wynn et al., 1995; Fig. 3). IFN- $\gamma$  inhibits fibrosis, whereas the Th2-associated cytokines IL-4, IL-5, and IL-13 have been causally linked to the development of fibrosis in a variety of chronic inflammatory diseases (Wynn, 2004). Transgenic mice that specifically over-express IL-4 or IL-13 in the lung confirmed that both cytokines function as profibrotic mediators by both directly and indirectly influencing the activation of myofibroblasts (Rankin et al., 1996; Zhu et al., 1999). IL-5 can also promote fibrosis in the lung by recruiting eosinophils that produce TGF- $\beta$ 1, PDGF, and IL-13 (Huaux et al., 2003b; Cho et al., 2004; Fulkerson et al., 2006; Reiman et al., 2006). Nevertheless, detailed mechanistic studies conducted with IL-4 and IL-13 inhibitors and *il-4*<sup>-/-</sup>, *il13*<sup>-/-</sup>, *il-4ra*<sup>-/-</sup>, and *il13ra1*<sup>-/-</sup> mice suggest that the IL-13 signaling pathway likely serves as the dominant inducer of Th2-dependent fibrosis in several chronic lung diseases (Chiaromonte et al., 1999b; Kumar et al., 2002; Huaux et al., 2003a; Kolodsick et al., 2004; Yang et al., 2004; Lama et al., 2006; Keane et al., 2007; Ramalingam et al., 2008). IL-13 is detected in the BAL fluid of IPF patients, IPF fibroblasts are hyperresponsive to IL-13, and expression of both IL-13



**Figure 3. Specialized subsets of T helper cells and macrophages play distinct roles in pulmonary fibrosis.**

After injury, epithelial cells release IL-25, IL-33, and TSLP, which can facilitate the development of profibrotic Th2 responses. T cells also release IL-21 and IL-25, which promote Th2 differentiation. Th2 cells release IL-4 and IL-13, which promote the development of a profibrotic macrophage subpopulation that secretes TGF- $\beta$ 1 among other mediators. IL-13 can also directly activate fibroblasts independently of TGF- $\beta$ 1. Th2 cytokines also trigger specific chemokines that promote the recruitment of collagen-secreting fibrocytes from the bone marrow, which amplify fibrotic responses. The resulting myofibroblasts release ECM components. However, Th2 cytokines can also trigger antifibrotic feedback mechanisms. For example, Th2 cytokines activate arginase-1 activity in M2 macrophages, which inhibit fur-

ther IL-13 production and myofibroblast differentiation. IL-13 can also up-regulate the IL-13 decoy receptor in fibroblasts, which antagonizes ECM production via a negative feedback loop. In addition, IFN- $\gamma$ , produced by Th1 cells, exhibits potent antifibrotic activity by suppressing collagen synthesis in fibroblasts and by promoting the activation of inflammatory M1 macrophages that favor ECM degradation. [Interactive PPT slides for this figure are available online.](#)

and IL-13R $\alpha$ 1 correlate with the severity of the disease (Murray et al., 2008; Park et al., 2009).

Consequently, recent studies have focused on elucidating mechanisms that regulate IL-13 effector function. In addition to expressing an IL-13 signaling receptor, a heterodimer composed of the IL-4R $\alpha$  and IL-13R $\alpha$ 1 subunits, fibroblasts also express a decoy receptor for IL-13, IL-13R $\alpha$ 2 (Chiaromonte et al., 2003). The latter exhibits four orders of magnitude increased affinity for IL-13 (Lupardus et al., 2010) and suppresses IL-13-IL-13R $\alpha$ 1-induced responses, including pulmonary fibrosis (Wilson et al., 2007; Ramalingam et al., 2008; Zheng et al., 2008). IL-21 produced by T cells, IL-33, and thymic stromal lymphopoietin (TSLP) released from damaged epithelial cells, as well as IL-25 produced by both T cells and epithelial cells, also represent potential targets for antifibrotic therapy, as each of these cytokines has been reported to play a major role in the induction and/or amplification of type 2 immunity (Fort et al., 2001; Schmitz et al., 2005; Zhou et al., 2005; Pesce et al., 2006). Nevertheless, although IL-21 regulates liver fibrosis during *S. mansoni* infection (Pesce et al., 2006) and IL-33 is a major inducer of IL-13-dependent cutaneous fibrosis (Rankin et al., 2010), few studies have examined whether these “Th2-initiating cytokines” participate in the development of pulmonary fibrosis (Ramalingam et al., 2009). In addition to focusing on upstream mediators that regulate IL-13 production, several groups have also been elucidating the downstream mechanisms that are targeted by IL-13. IL-1 (Ingram et al., 2004), TGF- $\beta$ 1 (Lee et al., 2001), adenosine (Blackburn et al., 2003), chitinase family members (Zhu et al., 2004; Lee et al., 2009), chemokines (Belperio et al., 2002a; Zhu et al., 2002; Ma et al., 2004), matrix metalloproteinases (Lanone et al., 2002; Madala et al., 2010) and various fibroblast subsets (Chiaromonte et al., 1999a; Murray et al., 2008; Shao et al., 2008) are emerging as important targets of profibrotic IL-13. Together, these studies support recent efforts investigating the therapeutic potential of IL-13 antagonists in IPF and other chronic lung diseases (Hariyawasam et al., 2009).

#### Chemokines recruit leukocytes and fibroblasts to the lung

Chemokines are a large family of chemoattractants that recruit leukocytes, fibroblast precursors, and other key effector cells to sites of tissue injury and therefore represent potential targets for antifibrotic therapy (Strieter et al., 2007). Serum levels of CCL18 correlate negatively with declining pulmonary function in patients with pulmonary fibrosis of various causes, suggesting that CCL18 might be developed as a serum biomarker of progressive pulmonary fibrosis (Prasse et al., 2006; Prasse et al., 2009).

Blocking or genetically deleting CCL2 (monocyte-chemoattractant protein-1), CCL6 (C10), or CCR1 provides significant protection from bleomycin-induced pulmonary fibrosis, albeit through mechanisms that remain undefined (Smith et al., 1994; Moore et al., 2001; Tokuda et al., 2000; Belperio et al., 2002a). CXCL12, CCL12, and CCR2 also play important roles in FITC- and bleomycin-induced pulmonary fibrosis

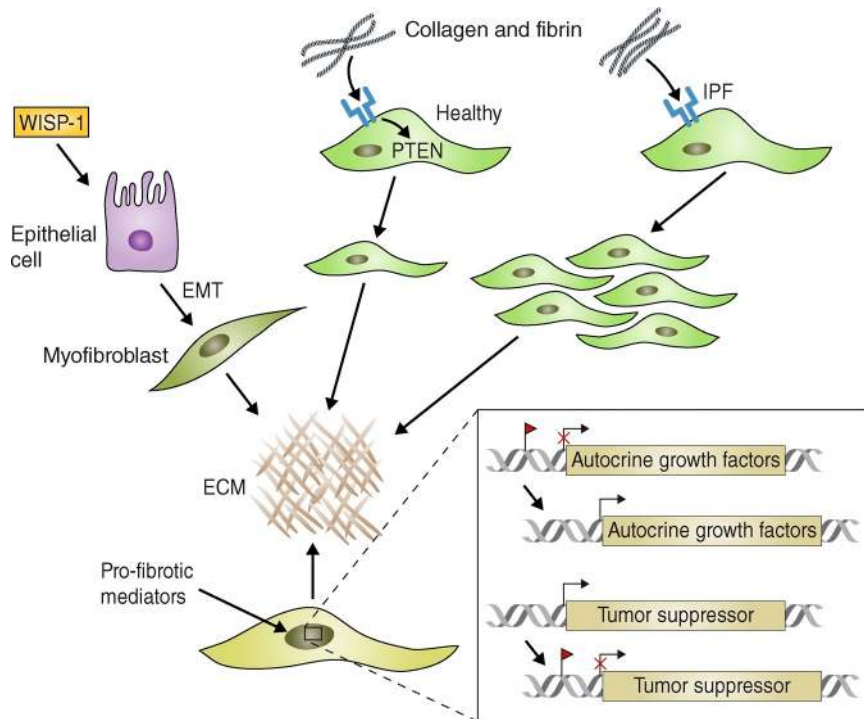
by facilitating the recruitment of bone marrow-derived collagen-secreting fibrocytes to the lung (Phillips et al., 2004; Moore et al., 2005, 2006). Signals through the chemokine receptors CCR2, CXCR3, and CXCR2 also regulate fibrosis associated with bronchiolitis obliterans syndrome, by regulating vascular remodeling and recruitment of mononuclear phagocytes and neutrophils (Belperio et al., 2001, 2002b, 2005). The lysophosphatidic acid receptor 1 also controls the recruitment of fibroblasts in mice with bleomycin-induced fibrosis and levels of lysophosphatidic acid are increased in the BAL fluid of people with IPF (Tager et al., 2008).

Interestingly, however, not all chemoattractants promote pulmonary fibrosis. CXCL10, CXCL11, and CXCR3 inhibit bleomycin-induced fibrosis by preventing fibroblast recruitment, decreasing angiogenesis in the lung, and by promoting the production of the antifibrotic cytokine IFN- $\gamma$ , respectively (Jiang et al., 2004, 2010; Burdick et al., 2005). Thus, disrupting and/or augmenting specific chemokine signaling pathways could have a significant impact on the progression of pulmonary fibrosis. Given the well-known redundancy in chemokine-chemokine receptor signaling, it will be important to determine which forms and stages of pulmonary fibrosis might benefit from targeting these pathways.

#### Factors influencing epithelial cell and fibroblast differentiation and proliferation

Aberrant activation of developmental and wound-healing pathways also contributes to the pathogenesis of pulmonary fibrosis, particularly in IPF, where ongoing inflammation is believed to play less of a role. For example, the Wnt- $\beta$ -catenin signaling pathway, which regulates homeostatic self-renewal in several adult tissues, is constitutively active in ATII cells in both a mouse model of pulmonary fibrosis and in patients diagnosed with IPF (Chilosi et al., 2003; Königshoff et al., 2008, 2009). Treatment with WNT1-inducible signaling protein-1 (WISP-1) promotes proliferation and EMT of mouse ATII cells and synthesis of ECM components by mouse and human lung fibroblasts. Development of pulmonary fibrosis in bleomycin-treated mice is reduced when the WNT1 pathway is blocked with WISP1-neutralizing antibodies (Königshoff et al., 2009). Thus, as an inducer of EMT, WISP1 is a potential therapeutic target in IPF (Fig. 4; Thiery and Sleeman, 2006). The integrin  $\alpha$ 3 $\beta$ 1, expressed on epithelial cells, is another important inducer of EMT. Therefore, the  $\alpha$ 3 $\beta$ 1 and Wnt- $\beta$ -catenin pathways both appear to be involved in the development of pulmonary fibrosis (Kim et al., 2009a).

Fibroblasts isolated from IPF patients also display constitutive changes in their proliferative ability (Ramos et al., 2001), fail to invade the ECM, and display altered expression of genes in matrix production and degradation (Lovgren et al., 2011), suggesting that intrinsic defects in the activation status of fibroblasts might contribute to the pathogenesis of pulmonary fibrosis.  $\beta$ 1 integrin inhibits fibroblast proliferation when bound to polymerized type 1 collagen by facilitating the activation of the tumor suppressor phosphatase and tensin homologue (PTEN), which normally suppresses the



**Figure 4. Intrinsic changes in the activation status of epithelial cells and fibroblasts can promote growth factor-independent pulmonary fibrosis.** Wnt- $\beta$ -catenin signaling activated (for example) by WISP-1, is constitutively active in some A11 epithelial cells in IPF patients and in mice with bleomycin-induced pulmonary fibrosis. This signaling triggers EMT and synthesis of ECM components by fibroblasts. In healthy fibroblasts, collagen-mediated stimulation of  $\beta_1$  integrin (blue) up-regulates PTEN activity and inhibits proliferation. IPF fibroblasts, however, display a pathological pattern of  $\beta_1$  integrin expression and signaling that can lead to decreased PTEN expression, aberrant activation of PI3 kinase, and excessive proliferation. Profibrotic mediators also promote epigenetic changes in fibroblasts that contribute to the pathogenesis of fibrosis. For example, the promoter regions of various genes encoding autocrine growth and/or differentiation factors can be demethylated, leading to their sustained and heritable activation. In addition, tumor suppressor genes can become methylated (red flag) and therefore inactivated, leading to the sustained activation of oncogenes that promote growth factor-independent proliferation of fibroblasts. miRNAs (e.g., miR-21) may operate in a similar fashion by blocking the translation or promoting the degradation of tumor suppressor genes in fibroblasts.

PI3K–Akt–S6K1 signaling pathway (Xia et al., 2008). IPF fibroblasts evade this inhibitory mechanism by displaying a pathological pattern of  $\beta_1$  integrin expression that leads to low PTEN activity and uncontrolled activation of the PI3K–AKT–S6K1 pathway (Fig. 4). In support of this theory, mice deficient in PTEN display a prolonged fibroproliferative response after tissue injury (Xia et al., 2008). Loss of either  $\beta$ -arrestin1 or  $\beta$ -arrestin2 was shown to block fibroblast invasion into the ECM and to protect mice from bleomycin-induced fibrosis (Lovgren et al., 2011). In contrast, mice deficient in the cationic amino acid transporter Slc7a2 exhibit heightened fibroproliferative responses and increased Th2-cytokine-associated fibrosis (Thompson et al., 2008). Thus, intrinsic defects in fibroblast proliferative pathways can have a significant impact on the progression of pulmonary fibrosis.

Epigenetic changes in fibroblasts have also been hypothesized to contribute to the pathogenesis of fibrosis by preventing proliferating fibroblasts from returning to their resting state. A recent genome-wide methylation scan of fibroblasts revealed several DNA methylation modifications that were unique to collagen-secreting myofibroblasts obtained from fibrotic kidneys (Bechtel et al., 2010). One of these modifications led to the epigenetic silencing of *Rasal1*, a suppressor of the Ras protooncogene, which led to increased Ras activity and growth factor-independent proliferation of fibroblasts. This study was important because it provided a novel molecular explanation for the sustained and heritable activation of fibroblasts that is often observed when fibrosis becomes advanced (Fig. 4). The targeted repression of known antifibrotic genes by hypermethylation may also contribute to the

development of pulmonary fibrosis (Coward et al., 2010). Interestingly, DNA methyltransferase inhibitors reversed epigenetic modifications and protected mice from pulmonary fibrosis (Sanders et al., 2008). Therefore, because epigenetic modifications are potentially reversible, they may represent attractive targets for novel antifibrotic therapies.

#### Additional potential therapeutic targets and strategies

Peroxisome proliferator-activated receptors (PPARs)  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  are ligand-activated transcription factors that belong to the nuclear hormone receptor family and important regulators of metabolic and inflammatory processes (Kostadinova et al., 2005). The receptors are found on a wide variety of tissues in the lung, including airway epithelial cells and smooth muscle cells, and PPAR signaling has been implicated in the pathogenesis of a wide variety of inflammatory diseases of the lung (Huang et al., 2005). Agonists of the three receptors suppress inflammation by inhibiting the production of proinflammatory cytokines, including IL-1 $\beta$  and TNF, and by reducing the influx of neutrophils into the lung. Some PPAR ligands also reduce the expression of Th2 cytokines, adhesion molecules, and chemokines that function as major drivers of pulmonary fibrosis (Sime, 2008). PPAR $\alpha$  agonists reduce the development of bleomycin-induced pulmonary fibrosis, and mice deficient in PPAR $\alpha$  develop exacerbated lung fibrosis associated with increased production of IL-1 $\beta$  and TNF (Genovese et al., 2005b). PPAR $\beta/\delta$  agonists also inhibit lung fibroblast proliferation and enhance the antifibrotic properties of PPAR $\gamma$  agonists. PPAR $\gamma$  agonists display



similar protective activity and inhibit TGF- $\beta$ 1-driven myofibroblast differentiation in vitro and the profibrotic activity of TGF- $\beta$ 1 in vivo (Burgess et al., 2005; Genovese et al., 2005a; Milam et al., 2008). Thus, in cases where pulmonary fibrosis is associated with a persistent proinflammatory response, PPAR ligands may prove beneficial.

MicroRNAs (miRNAs) are small, evolutionarily conserved, noncoding RNAs  $\sim$ 22 nt in length that play important roles in a variety of pathophysiologic processes by blocking the translation or promoting the degradation of specific target mRNAs. Unique miRNA expression patterns have been identified in a variety of lung disorders including COPD, emphysema, cystic fibrosis, asthma, lung cancer, and IPF, suggesting that distinct subsets of genes are targeted by microRNAs in each disease (Nana-Sinkam et al., 2009). Recently, miR-21 was identified in the lungs of patients with IPF and in mice with bleomycin-induced pulmonary fibrosis (Liu et al., 2010). In agreement with related studies (Thum et al., 2008; Kim et al., 2009b), miR-21 production was primarily localized to myofibroblasts and expression was tightly controlled by the profibrotic cytokine TGF- $\beta$ 1 (Liu et al., 2010). Administration of miR-21 antisense probes decreased the severity of bleomycin-induced fibrosis in mice and attenuated the profibrotic activity of TGF- $\beta$ 1 in fibroblasts, confirming a critical role for miR-21 in lung fibrogenesis. Interestingly, miR-21 targets several tumor suppressor genes and promotes tumor growth and invasion (Fig. 4), suggesting it can function as an oncogene (Meng et al., 2007; Zhu et al., 2007, 2008; Asangani et al., 2008). miR-21 also operates as antiapoptotic factor in tumor cells (Chan et al., 2005). Thus, aberrant expression of miR-21 in fibroblasts could promote their survival and differentiation into pathogenic collagen-secreting myofibroblasts. Therefore, small-molecule inhibitors of miR-21 might be developed to treat IPF. Identifying specific miRNAs that block profibrotic genes or promote lung regeneration could also prove highly beneficial in the treatment of pulmonary fibrosis (Pandit et al., 2010).

#### Disease stage-specific roles of macrophages

Macrophages are integrated into all stages of the fibrotic process, perhaps because they serve as key regulators of fibroblast recruitment, proliferation, and activation (Wynn and Barron, 2010). They promote fibrosis by secreting chemokines and specific matrix metalloproteinases that degrade ECM components, thus facilitating the recruitment of inflammatory cells to sites of tissue injury (Zuo et al., 2002; Jiang et al., 2005). They also produce several profibrotic mediators, including TGF- $\beta$ 1 and PDGF that induce the proliferation and activation of collagen-secreting myofibroblasts (Song et al., 2000). Alveolar macrophages isolated from fibrotic lung tissues are also capable of producing profibrotic cytokines (Hancock et al., 1998; Ingram et al., 2004; Cassel et al., 2008; Wilson et al., 2010).

Nevertheless, although some macrophages clearly promote tissue fibrogenesis, other macrophage subpopulations may facilitate the resolution and/or reversal of fibrosis

(Iredale, 2007; Wynn and Barron, 2010). Studies conducted with CD11b-DTR mice showed that if macrophages were depleted during the early inflammatory/maintenance phase of a fibrotic response, scarring was reduced and myofibroblasts were decreased (Fig. 1). In contrast, if macrophages were depleted during the late remodeling/recovery phase, fibrosis persisted (Duffield et al., 2005). This important study confirmed that macrophages could play distinct roles in the inflammatory and remodeling phases of wound healing and fibrosis. Macrophages inhibit fibrosis by secreting mediators that induce myofibroblast apoptosis, removing cellular debris that can drive inflammation, digesting and engulfing ECM components, and stimulating the production of collagen-degrading MMPs in a variety of cell types, including myofibroblasts and neutrophils (Atabai et al., 2009; Wynn and Barron, 2010). Th2 cytokine-stimulated macrophages that express the enzyme arginase-1 also exhibit potent antifibrotic activity (Pesce et al., 2009). An important goal of future research will be to determine if the pro- and antifibrotic activities of macrophages are performed by distinct subpopulations or whether the same macrophage can adjust its phenotype over time in coordination with new stimuli found in the local environment. Harnessing the protective activity of antifibrotic macrophages may be key to ameliorating established and progressive fibrosis, as restoration of normal tissue architecture can proceed only if the existing collagen matrix is successfully removed.

#### Conclusions and future perspectives

A variety of experimental models have been generated to study the mechanisms of pulmonary fibrosis (Moore and Hogaboam, 2008). However, the mouse bleomycin model has garnered the most attention, perhaps because it is a well-characterized and clinically relevant model of pulmonary fibrosis. Nevertheless, although it successfully models the early proinflammatory stages of the disease, because of the transient nature of the bleomycin response and the reversibility of the fibrosis, it is unclear whether this model can truly replicate the chronic and progressive forms of the disease seen in humans. Epithelial damage, inflammation, EMT, myofibroblast activation, and repetitive cycles of tissue injury are certainly important initiators of fibrosis. However, if we are to develop effective therapeutics for pulmonary fibrosis, a more detailed understanding of the complex environmental, cellular, genetic, and epigenetic changes that synergize to promote the progression of chronic pulmonary fibrosis is needed. Inflammatory and profibrotic mediators likely serve as the trigger for the epigenetic modifications that are observed in epithelial cells, endothelial cells, and fibroblasts. Therefore, a more integrated approach that targets key inflammatory cytokines, profibrotic mediators, and epigenetic modifications simultaneously will likely emerge as the most successful strategy to treat this highly complex and devastating disease. As there are numerous causes, forms, and stages of pulmonary fibrosis, the heterogeneous nature of the disease must be considered when

evaluating the results from individual mouse models, and, most importantly, when designing and implementing novel treatment strategies.

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