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TRANSLATIONAL REVIEW

Immune mechanisms in pulmonary fibrosis

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At a Glance Commentary: In this review, we discuss the emerging role of immune cells (T cells, neutrophils, macrophages and fibrocytes) and soluble mediators (cytokines and chemokines) involved in pulmonary fibrosis, pointing towards novel immune-based therapeutic strategies in the field.

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ABSTRACT

Pulmonary fibrosis, particularly idiopathic pulmonary fibrosis, represents a chronic and progressive disease with high mortality and limited therapeutic options. Excessive deposition of extracellular matrix proteins results in fibrotic remodeling, alveolar destruction and irreversible loss of lung function. Both innate and adaptive immune mechanisms contribute to fibrogenesis at several cellular and non-cellular levels. Here, we summarize and discuss the role of immune cells (T cells, neutrophils, macrophages and fibrocytes) and soluble mediators (cytokines and chemokines) involved in pulmonary fibrosis, pointing towards novel immune-based therapeutic strategies in the field.

Keywords: Fibrosis, immunity, lung, T cells, cytokines, chemokines, macrophages, fibrocytes, neutrophils

Word count: 87

I. PULMONARY FIBROSIS

Pulmonary fibrosis represents a chronic and progressive tissue repair response, which leads to irreversible scarring and remodeling of the lung. The fibrogenic triggers that initiate and maintain fibrotic pulmonary remodeling remain controversially discussed, but probably include infections (1), cigarette smoke (2), radiotherapy (3), chemotherapy (4), environmental and occupational pollutants (5, 6), obesity (7), diabetes mellitus (8), gastroesophageal reflux (8), pulmonary hypertension (9), obstructive sleep apnoea (10), chronic graft-versus-host disease (11) and connective tissue diseases/autoimmune disorders (12), such as rheumatoid arthritis (13), scleroderma (14) or Sjögren`s syndrome (15). However, pulmonary fibrosis can also manifest without any known etiology. Idiopathic pulmonary fibrosis (IPF) is the prototypic age-related and irreversible fibrotic disease with a median survival of 2–6 years following diagnosis and is largely refractory to current pharmacological treatments (16). To date, the highest genetic risk factor to develop IPF is a polymorphism in the MUC5B gene (17-19). Lung transplantation is the only effective treatment approach for IPF patients (20).

Fibrogenesis is thought to represent dysregulated and perpetuated wound healing / connective tissue repair in response to recurring alveolar microinjuries. A hallmark of this fibrotic repair process is the excessive deposition of extracellular matrix (ECM) components, such as hyaluronan, fibronectin, and interstitial collagens, which irreversibly remodel the lung tissue structure, leading to thickening of the alveolar and peribronchial walls, thus impairing gas exchange (21, 22). During wound healing, fibroblasts are key cells responsible for the synthesis and deposition of ECM by providing an initial scaffold for tissue regeneration (21, 22). When aberrant wound healing and fibrosis develops, fibroblasts respond by hyper-proliferating at sites of injury, acquire a “pro-fibrotic” phenotype resistant to apoptosis, and differentiate into highly contractile myofibroblasts that perpetuate the fibrotic process (21, 22). This activated fibroblast/myofibroblast is highly responsive to growth factors/cytokines, such as connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF),

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transforming growth factor-beta (TGF-β)1, interleukin (IL)-1β, IL-6, IL-13, IL-33 (23), as well as aberrantly activated pro-fibrotic pathways including TGF-β (24), Wnt (25), Sonic Hedgehog (26, 27), or Notch (28) that maintain fibrotic tissue transformation. Furthermore, recent work showed that the interplay between perivascular fibroblasts, epithelial cells, endothelial cells, and perivascular macrophages regulates the fine-tuning between alveolar repair and fibrosis through Wnt and Notch signaling interaction (29). Concisely, endothelial expression of CXC chemokine receptor (CXCR) 7 prevents epithelial damage by Jagged1 inhibition, whereas recruitment of vascular endothelial growth factor (VEGF) R1 expressing macrophages stimulates Wnt/β-catenin-dependent upregulation of Jagged1, thereby stimulating Notch signaling in fibroblasts and enhancing fibrosis (29).

Recently, subtypes of skin fibroblasts with intrinsic fibrogenic potential that express engrailed-1 (En1) were identified. These fibroblasts trigger increased ECM deposition during development and repair, and contribute to tissue fibrosis in multiple mouse models (30). CD26/DPP4 was identified as a surface marker of En1-positive fibroblasts. Depletion of En1-positive fibroblasts or small molecule-based inhibition of CD26/DPP4, leads to decreased connective tissue deposition and fibrosis (30).

Proteases play a key role in ECM remodeling (31). Particularly, matrix metalloproteinases (MMPs) and their inhibitor tissue inhibitors of metalloproteinases (TIMP)-1 have been involved in the pathogenesis of IPF and sarcoidosis (32). MMPs and TIMPs, mainly derived from macrophages, can either act pro- or anti-fibrotic, depending on the protease/antiprotease net balance and the microenvironmental tissue context (33-35). MMP-3 was found to initiate EMT in IPF by activation of the β-catenin signaling pathway through cleavage of E-cadherin (36). Gene expression studies further provided evidence for an upregulation and potential role of MMP-1, MMP-2, MMP-7 and MMP-9 in IPF (37, 38). Lung epithelial cells are critically involved in fibrogenesis through a sequence ranging from early epithelial damage to fibrogenic epithelial-mesenchymal transition (EMT) (39). EMT

renders epithelial cells to lose their canonical features, particularly cell-to-cell adherence, and to acquire migratory and mesenchymal properties, increasing their capability to convert to fibroblasts and to finally undergo transdifferentiation into myofibroblasts that synthesize ECM (40). During EMT, epithelial cells also lose their distinct marker expression profile, including E-cadherin, thyroid transcription factor-1, aquaporin-5, zonula occludens-1, and cytokeratins and acquire a mesenchymal morphology associated with expression of fibroblastic markers, particularly fibronectin extra domain A, alpha-smooth muscle actin (α-SMA), type I and III collagen, connective tissue growth factor, vimentin, and desmin (39-41).

Among the cytokines studied so far, primarily the pro-fibrotic cytokine transforming growth factor (TGF)-β1 has been described to play a central role in promoting EMT (22, 24). TGF-β1 drives EMT via SMAD2/3-dependent downstream mechanisms (42, 43) and promotes the transition of epithelial cells to fibroblasts through the transcription factors SNAI and TWIST (44, 45). Inflammation has a modulatory effect on TGF-β1-mediated pathways, as the pro-inflammatory cytokines IL-1β, tumor necrosis factors (TNF)-α and interferon (IFN)-γ were found to enhance TGF-β1-induced EMT via up-regulation of TGF-β receptor type I (46). Furthermore, the DAMP/alarmin High-Mobility Group Box 1 (HMGB1), released upon tissue injury by necrotic cells, enhanced EMT through the TGF-β1/SMAD2/3 pathway (47). The integrin α3β1, expressed on epithelial cells, phosphorylates β-catenin and activates pβ-catenin to form a complex with SMAD2 to initiate EMT (48). Li et al. further showed that prostaglandin (PG)-E2 could modulate cell migration following EMT through activation of E prostanoid (EP) 2 and EP4 as well as inhibition of EP1 and EP3 receptors (49). Recently, it has been shown that p63-positive lung epithelial basal cells overlying fibroblastic foci could act as EMT progenitors (50). Other EMT inducers include cigarette smoke (51), radiation (52), oxidative stress (53), mechanical stretch (54) and IL-17A (55). In contrast, other studies, including lineage tracing approaches, found no evidence for EMT in fibrotic settings (56-60). Additional translational research studies are warranted to solve these discrepancies.

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Besides TGF-β, dysregulated activation of the WNT-1-inducible signaling protein (WISP-1) plays a key role in IPF (25). WISP1 promoted lung fibrogenesis by increasing the release of pro-fibrotic cytokines and proteases from the alveolar epithelium, including SPP1, MMP-7, MMP-9, and plasminogen activator inhibitor 1, as well as by inducing EMT and increasing collagen production by fibroblasts (25). Wnt1/β-catenin signaling further promoted human embryonic pulmonary fibroblast to convert into myofibroblasts and enhanced ECM deposition upon tissue injury (61). Lrp5, a WNT coreceptor, was identified as a driver of lung fibrosis in mice and a marker of pulmonary fibrosis disease severity in humans with IPF (62). Therapeutically, WNT/β-catenin pathway inhibitors reversed established fibrosis and significantly improved survival in bleomycin-induced pulmonary fibrosis (63, 64). Recently, Wang et al. showed that inhibition of WNT/β-catenin signaling promoted the differentiation of bone marrow-derived mesenchymal stem cells into alveolar type II epithelial cells and inhibited fibroblast-to-myofibroblast transdifferentiation as well as ECM accumulation in bleomycin-induced pulmonary fibrosis (65).

II. IMMUNE CELLS IN PULMONARY FIBROSIS

Both innate and adaptive immune cell responses have been linked to (myo)fibroblast biology and fibrogenesis. Figure 1 and Table 1 summarize the main effects reported for key adaptive (T cell subsets) and innate (macrophages, neutrophils) immune cell types (Figure 1, Table 1). The immune cell skewing in pulmonary fibrosis probably affects anti-microbial host defence functions and infection susceptibilities; a topic that is beyond the scope of this review and is discussed in reviews dedicated to fibrosis and infections (66). In the chapters below we discuss the main studies published to date on distinct immune cell subsets and their potential involvement in pulmonary fibrosis.

T cells

There is emerging evidence that a skewed T-helper cell type 1/ T-helper cell type 2 (Th1/Th2) balance plays a modulatory role during the inflammatory phase of pulmonary fibrosis (22, 67). Systemic depletion of T cells using anti-CD3 monoclonal antibodies dampened ECM accumulation and fibrosis in a murine model of bleomycin-induced pulmonary fibrosis (68). The Th1 cytokines IFN-γ and IL-12 attenuated fibrosis (69), while the prototypical Th2 cytokines IL-4, IL-5 and IL-13 have been linked to fibrogenesis (70, 71), leading to the view that Th1 responses are protective, while Th2 responses are harmful (22, 71). At the transcriptional level, overexpression of the Th2 transcription factor GATA-3 or inhibiting the Th1 transcription factor T-bet modulated pulmonary fibrosis (72, 73). While in one study Th17 cells (T cells characterized by production of IL-17) showed no direct impact on fibroblast/myofibroblast activation and ECM production (74), other studies supported a role for IL-17 and Th17 cells by demonstrating that blocking/neutralization of IL-17A delayed the progression and promoted the resolution of pulmonary fibrosis in different murine fibrosis models (55, 75, 76). The potential role of regulatory T cells (Tregs, $CD4^{+}CD25^{high}FOXP3^{+}$) in IPF remains controversial. While on the one hand increased Tregs were reported (77), others demonstrated a reduction in Tregs in peripheral blood and BAL of IPF patients (78). Other findings support a pro-fibrotic role of Tregs in early stages of pulmonary fibrosis by increasing TGF-β1 release and collagen deposition (79), whereas at late stages Tregs were found to dampen lung fibrosis (79). Xiong et al. showed that Tregs depletion protected from radiation-induced lung fibrosis by increasing Th17 responses and shifting the Th1/Th2 balance towards Th1 (80). Other studies, however, showed that Tregs attenuated fibrocyte recruitment and pulmonary fibrosis via suppression of fibroblast growth factor-9 and CXC chemokine ligand (CXCL) 12 (81, 82). Viewing these studies in combination, the potential role of Tregs in pulmonary fibrosis remains incompletely defined. Tregs can exert probably both anti- and pro-fibrotic roles, depending on the stage of pulmonary fibrosis and mutual

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interactions with other T cell subtypes, an issue requiring further investigations. Th9 and Th22 cells, T cell subsets producing IL-9 or IL-22, were also involved in fibrosis, with dual pro- and anti-fibrotic effects described for Th9 (83-85) and protective effects for Th22 (86). Particularly, IL-9 overexpression *in vivo* yielded pro-fibrotic effects associated with high collagen and fibronectin deposition in bronchial areas (83), whereas other studies provided evidence for an anti-fibrotic role of IL-9 by showing that IL-9 mitigated silica-induced lung fibrosis and type 2 immunity (84) and was protective in a bleomycin-induced lung fibrosis model through a PGE_2-dependent mechanism (85). Gamma-delta (γδ) T cells were found to attenuate fibrotic responses via production of CXCL10 (87). Collectively, the role of T cells in pulmonary fibrosis seems to be complex and substantially depending on the subtype of T cells.

Macrophages

Besides their role as antimicrobial phagocytes, alveolar macrophages have been involved in the pathogenesis of fibrotic lung diseases. Alveolar macrophages represent a potent source of pro-fibrotic cytokines (such as TGF-β1 and PDGF), chemokines and proteases (MMPs) (88). However, conditional depletion of TGF-β1 from macrophages did not affect fibrosis (89). Depending on their polarization, the local micromilieu and the stage of fibrotic disease, alveolar macrophages have been reported to exert both pro- or anti-fibrotic effects (22, 88, 90). Particularly, the two contrasting macrophage phenotypes M1 (classically activated) and M2 (alternatively activated) are keys in understanding the beneficial versus harmful roles of alveolar macrophages in fibrotic diseases (91, 92). The prototypical Th2 cytokines IL-4 and IL-13 induce M2 macrophage polarization, characterized by production of IL-10, arginase-1, FIZZ-1 and distinct chemokines, particularly CCL17 and CCL18 (93). While M2 macrophages accumulate in fibrotic lungs and have been broadly associated with pro-fibrotic activities (92), their precise functional role in fibrotic environments remains uncertain and

poorly understood. M2 macrophages were also linked to anti-fibrotic activities as they were found to break down ECM by employing MMP-10 (94). Furthermore, M1 macrophages have been associated with pro-fibrotic roles, as supported by *in vivo* depletion studies (95). Collagen was found to induce M2 macrophages via the pro-fibrotic chemokine CCL18, thereby feeding a positive loop between fibroblasts and alveolar macrophages (96, 97). Macrophage receptor with collagenous structure (MARCO) has been further involved in polarization of macrophages towards a pro-fibrotic M2 phenotype and promoting fibrotic responses to lung injury (98). Shp2, a cytoplasmic tyrosine phosphatase associated with IL-4Rα, inhibited JAK1/STAT6 signaling through its phosphatase activity, inhibited macrophage skewing toward M2 phenotype and prevented pulmonary fibrosis (99). A central pathway for macrophage infiltration, MMP production and promotion of pulmonary fibrosis, is the CC chemokine ligand (CCL) 2 and its receptor CCR2 (see 4.1 for details) (100). TNF-α has been reported to exert anti-fibrotic effects and to accelerate resolution of established pulmonary fibrosis by decreasing M2 macrophages, potentially due to CCR2 downregulation and/or increased susceptibility of M2 macrophages to TNF-α-induced apoptosis (101). On the other hand, arginase-1, expressed by M2 macrophages, showed potent anti-fibrotic activity during Th2-driven inflammatory responses through depleting L-arginine, an amino acid essential for $CD4^+$ T cell and myofibroblast proliferation (102). In other models, conditional depletion of the M2-associated arginase-1 from macrophages did not affect Th2-mediated lung inflammation (103). Depletion of macrophages/monocytes in an animal model of pulmonary fibrosis reduced ECM deposition and, conversely, adoptive transfer exacerbated fibrosis (104). The pro-fibrotic roles of macrophages are mainly associated with recruitment and activation of fibroblasts through TGF-β1 and PDGF secretion (88, 90, 105). Depending on the cellular and environmental context, macrophages are also able to produce TIMPs thereby inhibiting degradation of ECM (90, 105). Anti-fibrotic roles of macrophages are believed to be mediated by a variety of mechanisms, including scavenging pro-inflammatory cellular

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debris, digesting ECM components by activation of collagen-degrading MMPs and by secreting mediators that induce myofibroblast apoptosis (90, 106-108). In summary, several *in vitro* and *in vivo* studies have involved macrophages and their products in pulmonary fibrosis, yet the distinct beneficial vs harmful roles of specific M1/M2 phenotypes remain unclear and controversially discussed.

Neutrophils

Like macrophages, neutrophils are not just antibacterial effectors, but also shape their tissue environment by releasing proteases, oxidants, cytokines and chemokines (109). Neutrophils were found to be increased in bronchoalveolar lavage (BAL) fluid from IPF patients and were associated with early mortality (110). Consistently, levels of IL-8/CXCL8, a key chemotactic factor for neutrophils, were increased in human IPF (111) and neutrophil counts in IPF BAL fluid correlated with levels of granulocyte-colony stimulating factor (G-CSF), a key growth factor for neutrophils (112). Cytokeratin 19, a potential marker for alveolar epithelial injury, correlated with the number of neutrophils in BAL fluid of IPF patients (113). Airway neutrophils in IPF seem to be activated, as reflected by their main proteolytic product, neutrophil elastase (NE), which was increased in airway fluids from IPF patients (114). NE breaks down a variety of ECM proteins, including collagens (types I-IV), laminin, entactin, fibronectin and elastin and thereby orchestrates the outcome of pulmonary fibrosis (115, 116). NE-deficient mice showed attenuation of pulmonary fibrosis through impaired TGF-β activation (116). Likewise, Sivelestat, a NE inhibitor, ameliorated pulmonary fibrosis through abrogation of TGF-β activation and inflammatory cells recruitment to the lung (117). Recently, Gregory et al. extended these findings by demonstrating a significant reduction of fibroblast and myofibroblast accumulation in $NE^{-/-}$ mice, which were protected from asbestos-induced pulmonary fibrosis (118). Further studies showed that NE promoted fibroblast proliferation and enhanced myofibroblast differentiation (118). Besides serine proteases,

neutrophils are also a substantial source of MMPs, such as MMP-2, MMP-8 (collagenase 2) and MMP-9 (gelatinase B), which are involved in pulmonary fibrosis (32,119). The balance between MMPs and their anti-proteases (TIMPs) plays a critical role for accumulation or degradation of extracellular matrix in pulmonary fibrosis (32, 120). The depletion of neutrophils has been associated with a MMP-9/TIMP-1 imbalance, but did not alter the susceptibility to bleomycin-induced pulmonary fibrosis (121).

Fibrocytes

Traditionally, fibroblasts are regarded as mesenchymal tissue originating / resident cells, but recent studies have established the concept that circulating myeloid-derived cells, termed fibrocytes, can migrate into tissues and differentiate into fibroblasts and myofibroblasts (122). Furthermore, fibrocytes secrete paracrine factors which activate resident fibroblasts to promote lung fibrosis (123). Fibrocytes express myeloid markers, such as CD45 and CD34, the chemokine receptor CXCR4 and Collagen-1 (124). Fibrocytes produce ECM components (collagen I, collagen III, fibronectin and vimentin), cross-linking enzymes (lysyl oxidase (LOX) family), cytokines (TNF-α, IL-6, IL-8 and IL-10), chemokines (MIP-1α/β, MCP-1 and GROα), growth factors (VEGF, PDGF, GM-CSF and others) and various matrix metalloproteinases like MMP-9 (125-127). Moeller et al. demonstrated that circulating fibrocytes were elevated in IPF patients and represented a prognostic marker and an independent predictor of early mortality (128). CCL12 and CXCL12 were found to be involved in attracting circulatory fibrocytes to the site of pulmonary injury (129, 130). The neuronal guidance protein Slit2, secreted by fibroblasts, was found to inhibit fibrocyte differentiation and reduced bleomycin-induced pulmonary fibrosis in mice (131). Recently, novel immunoregulatory properties of fibrocytes have been established, by demonstrating that fibrocytes with myeloid-derived suppressor cells (MDSCs) characteristics accumulate in patients with metastatic cancer (132). MDSCs are basically referred to as monocytic or

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granulocytic/neutrophilic innate immune cells characterized by their potential to suppress T cells (133). The novel proposed subtype of MDSCs, fibrocytic MDSC (f-MDSC), was found to differentiate from umbilical cord blood precursors upon culture with GM-CSF/G-CSF (134). Further studies demonstrated that distinct factors, including $CD4^+$ T cells, IL-2, IL-4, IFN-γ, TNF-α, GM-CSF/G-CSF, Kruppel-like factor 4 and fibroblast-specific protein 1 transdifferentiated $Gr1^+$ MDSCs-like cells into ECM (collagen-type-I)-producing fibrocytes (134-136). Fibrocytic MDSCs were also found to expand Tregs (134).

III. CYTOKINES IN PULMONARY FIBROSIS

TGF-β

TGF-β is probably the best studied cytokine in fibrosis and is regarded as a prototypical 'pro-fibrotic' mediator (24). Among three isoforms, TGF-β1 has been mainly involved in pulmonary fibrosis (137). After dissociation from latency-associated protein, TGF-β1 increases the transcription of downstream target genes, including pro-collagen I and III via transmembrane receptor serine/threonine kinases and the cytoplasmic SMAD-2/3 signaling pathways (138). Particularly, SMAD-3 deficiency has been shown to ameliorate bleomycin-induced pulmonary fibrosis (139). Moreover, extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase, the phosphatidyl inositol 3-kinase/Akt pathway and Rho-like GTPase pathways have also been involved in TGF-β1-induced fibrosis (140-142). Mechanistically, TGF-β1 promotes ECM accumulation, especially collagen and fibronectin, and drives phenotypic changes of fibroblasts (143, 144). TGF-β1 differentiates fibroblasts into myofibroblasts by inducing expression of α-SMA (145). However, it has recently been shown that α-SMA-expressing myofibroblasts may not represent the only source of pathologic collagen deposition in fibrotic settings (146). Recently, another study showed that TGF-β1 increases vascular cell adhesion molecule 1 and promotes fibroblast proliferation in IPF patients (147). Furthermore, TGF-β1 enhances fibroblast proliferation and promotes

pulmonary fibrosis via BARD1 (BRCA1 Associated RING Domain 1) pathway (148). Galectin-3 is also involved in TGF-β1-induced pulmonary fibrosis by increasing EMT, myofibroblast activation and collagen production (149). Glycogen synthase kinase-3 regulates TGF-β1-induced fibroblast-to-myofibroblast differentiation via a cAMP response element-binding protein (CREB)-dependent mechanism (150). Furthermore, chitinase-1 has been involved in TGF-β1-induced pulmonary fibrosis by increasing TGF-β1 receptor expression (151). Recently, Oruqaj et al. showed that peroxisomes are involved in TGF-β-induced myofibroblast differentiation and collagen production in IPF (152).

PDGF

Besides TGF-β, PDGF represents another potent fibrogenic cytokine/growth factor that promotes pulmonary fibrosis through fibroblast activation (153). PDGF expression was found to be increased in epithelial cells and macrophages in the lungs of IPF patients (154). *In vivo*, pulmonary PDGF overexpression induced severe pulmonary fibrosis (155). PDGF acts through IP3-gated channels and increases $Ca2^{+}$ release to modulate ECM gene expression in human pulmonary fibroblasts (156). PDGF is a potent mitogen and chemoattractant for lung fibroblasts and acts through the PDGF receptor α (153). IL-13 was found to increase PDGF gene expression in lung fibroblast through STAT1 and STAT6 (157). Tregs promoted pulmonary fibrotic responses by stimulating fibroblasts through the secretion of PDGF in silica-induced pulmonary fibrosis (158). Imatinib, a PDGF tyrosine kinase inhibitor, showed strong anti-fibrotic effects in bleomycin-induced pulmonary fibrosis via inhibiting mesenchymal cells proliferation (159).

IL-1β

IL-1β, the primary cytokine product of the inflammasome, is mainly produced by activated macrophages, dendritic cells, neutrophils and epithelial cells and has been shown to contribute

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to the progression of pulmonary fibrosis (160). Expression of *IL-1β* mRNA was found to be up-regulated in bleomycin-induced pulmonary fibrosis (161) and overexpression of IL-1β in rat lungs promoted lung fibrosis characterized by the presence of myofibroblasts, fibroblast foci and ECM accumulation (160). Bleomycin-induced pulmonary fibrosis was attenuated in IL-1 receptor- or MyD88-deficient mice and exogenous recombinant IL-1β protein resembled bleomycin-induced lung pathology, corroborating a key role for IL-1 β in fibrogenesis *in vivo* (162). In BAL fluid and serum of IPF patients, the ratio of IL-1 receptor antagonist (IL-1Ra) and IL-1β was decreased (163). Several studies further involved the NLRP3 inflammasome in silica- and asbestos-induced pulmonary fibrosis (164, 165). Extracellular ATP, an activator of the NLRP3 inflammasome, was increased in BAL fluid of patients with IPF and in bleomycin-induced pulmonary fibrosis (166). Like ATP, the NALP3 inflammasome activator uric acid has been involved in bleomycin-induced pulmonary fibrosis (167). Wilson et al. further showed that IL-1β-induced pulmonary fibrosis is IL-17 dependent (168). The WNT/β-catenin signaling pathway was found to induce IL-1β expression by alveolar epithelial cells in pulmonary fibrosis (169).

IL-13

The Th2 cytokine IL-13 was found to be increased in the blood and BAL fluid of IPF patients and correlated with disease severity (170). IL-13 promoted pulmonary fibrosis in fluorescein isothiocyanate– and radiation-induced lung fibrosis models (171, 172), while IL-13 inhibition decreased fibrotic changes in pulmonary fibrosis model *in vivo* (173). IL-13-induced pulmonary fibrosis was reported as either TGF-β-dependent or -independent (174, 175). Mechanistically, IL-13 differentiates human lung fibroblast to myofibroblast through a JNK-dependent pathway (176). Downstream IL-13 effects were mediated through a complex receptor system that includes the IL-4 receptor (R) α, IL-13Rα1 and/or the IL-13Rα2 (177). IL-13-induced fibrosis was exaggerated when IL-13Rα2 was low or absent in target cells such

as fibroblasts (178). The transcription factor Yin Yang 1 (YY1) has been shown to directly regulate collagen and α-SMA expression in fibroblasts (179). IL-13, in turn, was found to stimulate fibroblasts and increase α-SMA through AKT-mediated YY1 activation (180).

IL-17

Previous studies linked IL-17 to pro-fibrotic effects, such as EMT and collagen production, through interactions with TGF-β signaling (55, 168). IL-17 inhibition attenuated pulmonary fibrosis via autophagic degradation of collagen and increased survival in bleomycin-induced lung fibrosis (55). Neutralizing IL-17 ameliorated progression of silica-induced lung fibrosis associated with delayed neutrophil recruitment, decreased Th17 cells, decreased IL-6/IL-1β production and increased Tregs (76). Neutrophils and monocytes/macrophages, rather than Th17 lymphocytes, were identified as cellular source of IL-17 and promoted pulmonary fibrosis in experimental hypersensitivity pneumonitis (181). It has been recently shown that B-cell activating factor (BAFF) was increased in BAL fluid of IPF patients (182). BAFF enhanced IL-17 release from Th17 cells and was involved in IL-17-induced pulmonary fibrosis (182). IL-27 attenuated pulmonary fibrosis by suppressing the secretion of IL-17 and the JAK/STAT and TGF-β1/SMAD signaling pathways (183). IL-17 production by γδ T cells in response to epithelial cell injury was mediated via IL-23 in pulmonary fibrosis (184).

IV. CHEMOKINES IN PULMONARY FIBROSIS

CCL2

CCL2 (MCP1), is produced by monocytes/macrophages, fibroblasts, and epithelial cells and acts via CCR2 (185). CCL2 was found to be increased in BAL fluid and serum of IPF patients (186) and murine pulmonary fibrosis studies showed that ECM deposition is attenuated in CCR2 knockout mice (185) and that this effect is linked to a reduction in macrophage infiltration and macrophage-derived MMP-2 and MMP-9 production (100). Moreover, CCL2

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increased fibrocyte recruitment to the alveolar space and promoted differentiation into fibroblasts, resulting in excessive collagen deposition (187). Proteinase-activated receptor-1, released from alveolar and bronchial epithelial cells, increased CCL2 release (188). The CCL2/CCR2 axis was further found to be involved in IL-10-induced macrophage and fibrocyte recruitment as well as M2 activation in pulmonary fibrosis (91). CCL2 stimulated IL-6 production by human lung fibroblasts through ERK1/2 signaling pathway and enhanced fibroblast survival by inhibiting apoptosis through IL-6/STAT3 in pulmonary fibrosis (189).

CCL17

Thymus and activation-regulated chemokine (TARC, CCL17) is constitutively expressed in the thymus and is inducible in PBMCs, macrophages, bronchial epithelial cells, endothelial cells and dendritic cells. CCL17 binds to CCR4 for its biological effects (190). CCL17 was found to be increased in both animals model of pulmonary fibrosis and human IPF patients and promoted fibrosis through the recruitment of $CCR4^+$ Th2 cells and alveolar macrophages (191-193). Neutralizing CCL17 could significantly ameliorate fibrosis progression *in vivo* (193). CCR4 was found to be highly expressed on T lymphocytes in the BAL fluid of IPF patients (194).

CCL18

The pro-fibrotic chemokine CCL18, previously known as pulmonary and activation-regulated chemokine (PARC), is produced by macrophages, dendritic cells, peripheral blood monocytes, eosinophils and neutrophils. CCL18 levels have been increased in serum, BAL fluid and sputum of IPF patients (97, 195, 196). IPF patients with a CCL18 serum cutoff level higher than 150 ng/ml showed an increased risk of mortality (195). Mechanistically, CCL18 increased collagen production in lung fibroblasts through different pathways, including ERK1/2, protein kinase Cα and Sp1/SMAD3 (197-199). After adenoviral gene transfer,

CCL18 promoted T cell infiltration and collagen accumulation in a mouse model of pulmonary fibrosis *in vivo* (200).

CXCL12

The CXCL12/CXCR4 axis has been involved in bleomycin-induced pulmonary fibrosis, as neutralizing CXCL12 dampened fibrocyte recruitment and pulmonary collagen deposition (130). Likewise, pharmacological CXCR4 antagonists alleviated bleomycin- and radiation-induced pulmonary fibrosis (201, 202). Bone marrow-derived lung $CXCR4^+$ cells were found to migrate in response to CXCL12 and differentiated to collagen-producing lung fibroblasts (203). In both familial and sporadic pulmonary fibrosis, gene expression of *CXCL12* was increased (204). Recently, Lin et al. showed that the CXCL12/CXCR4 axis activated the Rac1/ERK and JNK signaling pathways to induce activator protein-1 (AP-1) activation and CTGF expression in human lung fibroblasts (205). CTGF, in turn, mediated CXCL12-induced α-SMA expression and fibroblast differentiation to myofibroblasts (205).

V. THERAPEUTIC CONSEQUENCES

Anti-TGF-β1

TGF-β1 is potentially one of the main targets for treatment of pulmonary fibrosis (24, 206), since TGF-β1 inhibition showed anti-oxidant, anti-inflammatory and anti-fibrotic properties both in *in vitro* and *in vivo* models of pulmonary fibrosis (207, 208). Studies demonstrated that targeting TGF-β1 by monoclonal antibodies reduced pulmonary fibrosis in a murine model of bleomycin-induced pulmonary fibrosis (209). Targeting αvβ6 integrin, a key activator of TGF-β, also attenuated pulmonary fibrosis (210). Paclitaxel, an anti-tumor drug which stabilizes cellular microtubules, decreased TGF-β1/SMAD3 via upregulating miR-140 and ameliorated pulmonary fibrosis (211). Targeting the activin receptor–like kinase 5, a type I receptor of TGF-β that phosphorylates and activates SMADs, was further shown to inhibit

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pulmonary fibrosis (212). A TGF-β1 peptide inhibitor alleviated pulmonary fibrosis in murine model of bleomycin-induced pulmonary fibrosis through inhibition of fibroblast differentiation into myofibroblasts (213).

Anti-IL-13

Immunoneutralization of IL-13 attenuated pulmonary fibrosis in bleomycin-induced pulmonary fibrosis (214). Jakubzick et al. further demonstrated that an IL-13 immunotoxin chimeric molecule (IL13-PE) attenuated bleomycin-induced pulmonary fibrosis by reducing the number of IL-13- and IL-4-responsive cells (215). Recently, it has been further demonstrated that Tralokinumab, a human IL-13–neutralizing monoclonal antibody, dampened pulmonary fibrosis and promoted lung repair in a humanized SCID (severe combined immunodeficiency) IPF model (216).

New approaches

Pirfenidone

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) has been approved for the treatment of IPF in Japan in 2008 and later on in Europe, India, Canada, and recently in the USA (217). Although, to-date the exact mechanism of action of pirfenidone is poorly understood, pirfenidone shows evidence to attenuate lung fibrosis via inhibition of collagen synthesis and heat shock protein (HSP) 47 expression in lung fibroblasts (218), inhibition of pro-fibrotic and pro-inflammatory cytokines, including TGF-β1, IL-1β, IL-6 and b-FGF (219) and inhibition of fibrocyte migration via the attenuation of CCL2 and CCL12 production (220). Moreover, pirfenidone decreased human lung fibroblast proliferation and differentiation into myofibroblasts by inhibiting TGF-β-induced phosphorylation of SMAD3 (221), while it has been shown ineffective in reducing collagen secretion in primary human lung fibroblasts in another study (222). Although, the initial report of the clinical trials was not concordant,

CAPACITY-2, ASCEND, and a clinical trial performed in Japan demonstrated that pirfenidone slowed lung function decline and improved patient survival (223, 224). In these trials, pirfenidone was more effective in mild-to-moderate IPF patients, highlighting the importance of early diagnosis and treatment in pulmonary fibrosis (223, 224).

Nintedanib

Nintedanib (BIBF 1120), a triple-tyrosine kinase inhibitor, has been approved by the FDA at the same day as pirfenidone for IPF (225, 226). Nintedanib ameliorated progression of pulmonary fibrosis in murine models of silica- or bleomycin-induced pulmonary fibrosis (227, 228). Mechanistically, it has been shown that nintedanib inhibits FGF-, PDGF- and VEGF-induced pro-fibrotic effects, attenuates TGF-β-induced collagen deposition, reduces infiltration of inflammatory cells into the lungs and prevents TGF-β-induced human lung fibroblast differentiation to myofibroblast (222, 227-229). Nintedanib further potently blocked FGF receptor (R)1-3, PDGFR as well as VEGFR kinase activity (225) and modulated the protease/anti-protease balance (pro-MMP-2 and TIMP-2) (228). TOMORROW and INPULSIS studies showed that nintedanib slows lung function decline, decreases the frequency of short-term exacerbations and mortality as well as retains quality of life of patients with mild-to-moderate IPF (226, 230).

VI. CONCLUSIONS

Pulmonary fibrosis is a progressive, irreversible and usually lethal lung disease. Alveolar epithelial cell micro-injuries are thought to initiate the disease, followed by expansion of myofibroblasts and excessive deposition of extracellular matrix components that finally remodel and destroy the lung architecture. Immune mechanisms contribute to fibrogenesis at several cellular and non-cellular levels. In adaptive immunity, most evidence exists for T cells, whose role seems to be complex and subset-dependent. While Th1, Th22 and γδ-T cells have been proposed to attenuate pulmonary fibrosis, Th2 and Th17 cells were found to

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promote fibrotic disease. Tregs and Th9 subsets have been shown to exert both anti- and pro-fibrotic effects. Among innate immune cells, particularly M2 macrophages and neutrophils were suggested to enhance pulmonary fibrosis, whereas M1 macrophages were assigned a protective role, but also contradictory effects have been described and future studies are required to clearly define their roles *in vivo*. Fibrocytes represent bone marrow-derived immune cells that migrate to the lung and promote fibrosis. TGF-β, PDGF, IL-13, IL-17, IL-1β are the major cytokines and CCL2, CCL17, CCL18 and CXCL12 the main chemokines involved in the immunopathogenesis of pulmonary fibrosis. Targeting specific pro-fibrotic immune cell subsets (such as Th2/M2 cells) or pro-fibrotic cytokines/chemokines (such as TGF-β, IL-13, CCL2 or CCL18) by monoclonal antibodies or small molecules or expanding/activating anti-fibrotic cell types (such as Th1/M1 cells) may pave the way for novel immunopharmacological interventions for treating pulmonary fibrosis. Despite these intriguing insights, further studies are warranted to better understand the functional role of immune cell subtypes and their microenvironmental and contextual interaction with epithelial cells, (myo)fibroblasts and ECM components in the pathogenesis of pulmonary fibrosis.

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FIGURE LEGENDS

Figure 1. Effect of immune cells on myofibroblasts and fibrogenesis

Airway epithelial cell injury / epithelial-mesenchymal transition (EMT), fibroblast transdifferentation and/or chemokine (CCL2, CXCL12)-mediated fibrocyte recruitment contribute to the generation of myofibroblasts, which represent the major producers of extracellular matrix (ECM) components. Both adaptive (T cells, left box) and innate (macrophages and neutrophils, right box) immune cells modulate fibrogenesis through various mechanisms. Adaptive immunity: Th2 and Th17 cells promote pulmonary fibrosis, while Th1, Th22 and γδ-T cells inhibit fibrogenosis. Tregs and Th9 cells have been associated with both anti- and pro-fibrotic effects. Innate immunity: Macrophages might enhance pulmonary fibrosis through production of TGF-β and PDGF, or ameliorate pulmonary fibrosis by enhancing ECM degradation through matrix metalloprotease (MMP) activities. Macrophages further represent a source of tissue inhibitors of metalloproteinases (TIMPs) that can antagonize MMP-mediated ECM degradation. Neutrophils produce various proteases, particularly serine proteases (neutrophil elastase, NE) and MMPs, which degrade matrix components, but can also activate TGF-β through NE and produce TIMPs, thereby promoting ECM accumulation. Dashed lines represent effects/interactions that are complex/multifaceted or not firmly established.

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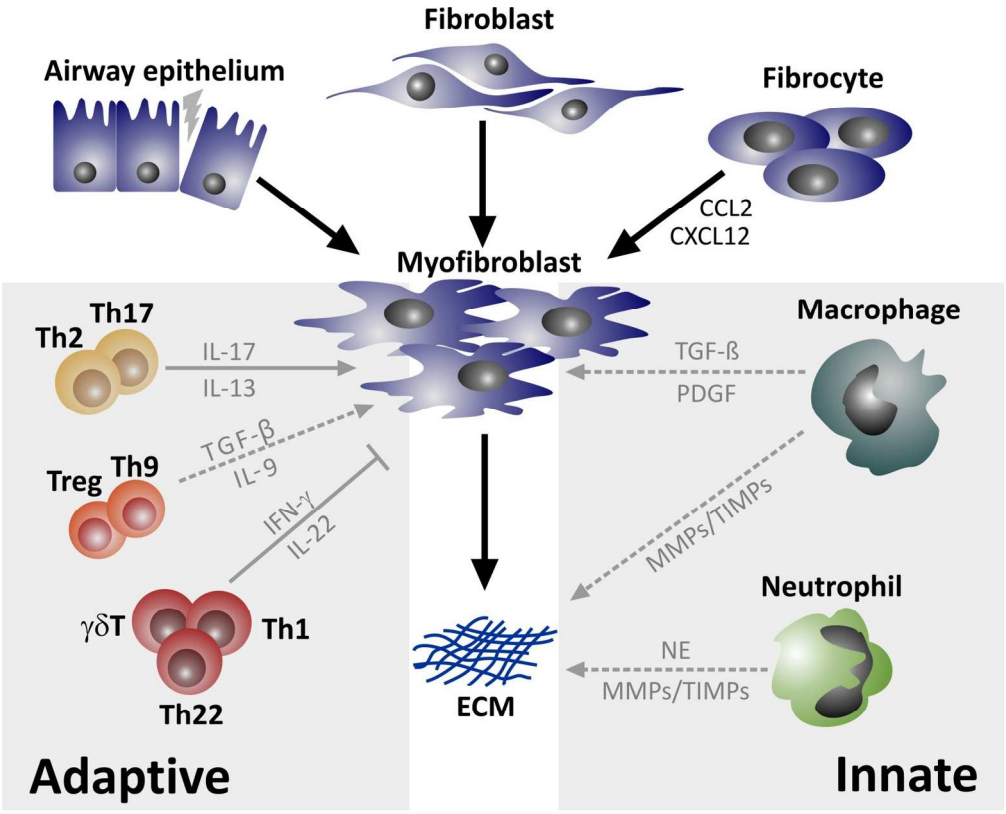

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156x125mm (300 x 300 DPI)

Table 1. Immune cells and mediators involvement in pulmonary fibrosis

Immune cells	
T cells	Th1 cytokines (IFN-γ and IL-12) attenuate PF, Th2 cytokines (IL-4, IL-5 and IL-13) enhance PF, Th17 cells enhance PF, Tregs and Th9 (IL-9) have both pro- and anti-fibrotic roles in PF, Th22 (IL-22) and γδ-T cells have anti-fibrotic role in PF.
Macrophages	M1 macrophages induce myofibroblast apoptosis and digest ECM by activation of MMPs. M2 macrophages recruit and activate fibroblasts through TGF-β1 and PDGF secretion. M2 macrophages further produce TIMPs and inhibit degradation of ECM. Both macrophage phenotypes (M1/M2) can exert pro- and anti-fibrotic effects.
Neutrophils	Neutrophils produce elastase, MMPs and TIMPs. Neutrophil elastase activates TGF-β and recruits inflammatory cells to the lung, thereby promoting PF.
Fibrocytes	Fibrocytes produce ECM, cross-linking enzymes, chemokines, growth factors and MMPs and promote PF. Fibrocytes secrete paracrine mediators which activate resident fibroblasts to promote PF. Fibrocytes can differentiate into fibroblasts and myofibroblasts.
Cytokines	
IL-1β	Pro-fibrotic effects of IL-1β, mediated through IL-1R1/MyD88 signaling pathway.
IL-13	IL-13 differentiates human lung fibroblast to myofibroblast through a JNK-dependent pathway.
IL-17	IL-17 interacts/cooperates with TGF-β signaling to promote PF.
TGF-β1	TGF-β promotes EMT through SMAD-2/3 signaling pathways. TGF-β1 induces PF through ERK, MAPK, PI3K/Akt and Rho-like GTPase Pathways. TGF-β1 differentiates fibroblasts into myofibroblasts and increases ECM accumulation.
PDGF	PDGF stimulates fibroblasts and increases ECM gene expression in fibroblasts.
Chemokines	
CCL2	CCL2 increases fibrocyte recruitment and differentiation into fibroblasts resulting in excessive collagen deposition. CCL2 activates M2 macrophage activation and promotes PF.
CCL17	CCL17 promotes PF through the recruitment of $CCR4^+$ Th2 cells and alveolar macrophages.
CCL18	CCL18 increases collagen production in lung fibroblasts through ERK1/2, PKCα and Sp1/Smad3 signaling pathways.
CXCL12	CXCL12 recruits fibrocytes and activates the Rac1/ERK and JNK signaling pathways to induce AP-1 activation and CTGF expression in fibroblasts.

PF=pulmonary fibrosis, Th1=T-helper cell type 1, Th2=T-helper cell type 2, IFN=interferon, IL=interleukin, ECM=extracellular matrix, MMP=matrix metalloproteinase, TIMP=tissue inhibitors of metalloproteinase, TGF=transforming growth factor, PDGF=platelet derived growth factor, WISP=WNT-inducible signaling protein, PAI=plasminogen activator inhibitor, CCL=CC-chemokine ligand, CXCL=CXC-chemokine ligand, AP-1=activator protein 1, CTGF=connective tissue growth factor