Title
Activation of JUN in fibroblasts promotes pro-fibrotic programme and modulates protective immunity

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Abstract

In pulmonary fibrosis, the transcription factor JUN is highly expressed in the fibrotic foci. Its induction in adult mice drives lung fibrosis, which is abrogated by administration of anti-CD47. Here, we use high-dimensional mass cytometry to profile protein expression and the secretome of individual fibroblasts and leukocytes from pulmonary fibrosis patients. We show that JUN is activated in fibroblasts derived from fibrotic lungs which also demonstrated increased CD47 and PD-L1 expression. Using ATAC-seq and ChIP-seq, we found that activation of JUN in fibroblasts rendered enhancers of CD47 and PD-L1 accessible, an observation that reporter assays corroborated. Meanwhile we detected increased IL-6 signaling which amplified both JUN-mediated CD47-enhancer activity and protein expression in fibrotic lung fibroblasts. Using an in vivo mouse model of fibrosis, we found two distinct mechanisms by which blocking IL-6, CD47, and PD-L1 reversed fibrosis—increased phagocytosis of profibrotic fibroblasts and elimination of suppressive effects on adaptive immunity. Our results identify specific immune mechanisms that promote the fibrotic process and suggest a complementary therapeutic approach that could be used alongside conventional anti-fibrotics for pulmonary fibrosis diseases.
Introduction

With a three-year survival rate of only 50%, pulmonary fibrosis has a terrible prognosis rivaling some of the worst malignancies. Pulmonary fibrosis is characterized by the spontaneous onset of progressive scarring of the lung in the absence of an infectious or autoimmune etiology\textsuperscript{1-5}. Despite the discovery that germline mutations of TERT are highly prevalent in these patients, the pathophysiological mechanism of pulmonary fibrosis disease remains incompletely understood. There are no curative treatments other than lung transplantation, thus, novel therapies are desperately needed\textsuperscript{6-10}. Clinical trials for nintedanib\textsuperscript{11,12} and pirfenidone\textsuperscript{13}, two standard-of-care treatments targeting the receptor tyrosine kinases VEGFR, FGFR and PDGFR, and the TGFB pathways, respectively, are known to play a role in idiopathic pulmonary fibrosis, despite trends in improvement in mortality rates with antifibrotics patients keep progressing and a high unmet need to halt progression remains. Other treatment strategies including inhibiting toll-like receptors (TLR3, 4, 9) and metalloproteases, blocking macrophage activation and recruitment (mAbs TNFa and CCL2), targeting Th1(=protective)/Th2(=profibrotic) imbalance with INFg and mAB IL-13, or immune modulatory treatments with CTLA4 and azathioprine in pulmonary fibrosis patients treated for cancer also failed to be effective in pulmonary fibrosis\textsuperscript{14-18}.

Fibroblasts are known to be at the core of the fibrotic response; however, quite surprisingly, they represent a poorly characterized cell type. Fibroblasts are heterogeneous, and no common consensus exists on their subtypes or biological properties such as signaling and plasticity. Recently, single-cell deconvolution of fibroblast heterogeneity was reported in a bleomycin-initiated pulmonary fibrosis mouse model\textsuperscript{19}. However, no comprehensive single-cell data focusing on fibroblasts are yet available for pulmonary fibrosis patients. We tested the most-reported canonical fibroblast markers\textsuperscript{20} and found that each only labeled a subset of fibroblasts. There are no universal fibroblast-specific markers. The level of heterogeneity with these so-called fibroblast markers could potentially bias analyses if only a single marker is selected to define fibroblasts. Furthermore, the mixed phenotype of certain fibroblasts like myofibroblasts, would also limit the extent of analyses if other mesenchymal
markers were included for negative gating. Hence, we decided to define fibroblasts by only excluding leukocytes (CD45), epithelial cells (CK7), and endothelial cells (CD31). This strategy not only helped us to enrich but also to further characterize the heterogeneity of fibroblasts in pulmonary fibrosis.

Monocytes and macrophages, as part of the innate immune response, are thought to play a critical role, regulating both injury and repair in various models of fibrosis. In addition, macrophage heterogeneity has emerged as an important area of study in fibrotic lung. Adaptive immune processes have been shown to orchestrate existing fibrotic responses and various subsets of T cells have been shown to be enriched in fibrotic lung. In addition, increased activated regulatory T cells correlate with the severity of fibrosis. Mass cytometry enables measurements of over 40 parameters simultaneously at the single-cell level when mass-tagged antibodies are used to label cellular proteins of interest and subsequently analyzed by time-of-flight mass spectrometry. Here, we used mass cytometry to characterize millions of primary lung cells from 11 pulmonary fibrosis patients and 3 normal donors at the single-cell level to determine the identities and functional aspects of various cell subsets in fibrotic lungs and systematically monitor interactions between cells in the microenvironment.

We previously showed that JUN caused severe lung fibrosis when induced in adult mice. This represents a non-chemical, purely genetic model of lung fibrosis and highlights one critical transcription factor at the core of a general fibrotic response. Moreover, the activated phosphorylated form of JUN could represent a new biomarker to predict poor outcome in lung fibrosis. We observed that JUN induction in mice resulted in upregulation of the CD47 protein in fibroblasts within less than 24 hours. CD47 is a key anti-phagocytic molecule that is known to render malignant cells resistant to programmed cell removal, or efferocytosis; it is a key driver of impaired cell removal. We were then able to demonstrate that we could prevent fibrosis in mice with anti-CD47 immune treatment. Importantly, now we also find that anti-CD47 immune therapy largely reverses the fibrotic reaction. However, the molecular details of how JUN caused, or CD47 blockade disrupted, the development of lung fibrosis and the implications for human pulmonary fibrosis diseases remained unknown.
Here, our single-cell protein screening approach in fibrotic lung patients highlighted two immune regulatory pathways dysregulated in fibrotic lung, CD47 and PD-1/PD-L1. Antibody therapies against both are currently being tested in clinical trials for cancer and recently have also been demonstrated to prevent atherosclerosis\textsuperscript{30-32}. In addition, we identified cytokine IL-6 at the core of progredient fibrosis in fibrotic lung. IL-6 is known to mediate its broad effects on immune cells (adaptive and innate) via a complicated signaling cascade in an almost hormone-like fashion, e.g., \textit{in vitro} experiments demonstrated that lung macrophages produce soluble IL-6Ra, and that increased IL-6 signaling increased extracellular matrix production. A clinically tested blocking antibody against IL-6 is available and FDA approved for rheumatoid arthritis\textsuperscript{33,34}.

\textbf{Results}

\textbf{The immune checkpoint proteins PD-L1 and CD47 are upregulated in fibroblasts in pulmonary fibrosis.}  

To systematically profile the pathophysiology of human pulmonary fibrosis, we applied an -omics approach combining multi-parameter single-cell mass cytometry and genome-wide chromatin accessibility assays together with a multiplexed Luminex secretome analysis as outlined in (Fig. 1a). For profiling with mass cytometry, single-cell suspensions of 14 representative lung samples, 11 fibrotic and 3 normal (all clinical information has been provided in Supplementary Table 1), were stained with a panel of 41 metal-conjugated antibodies (Supplementary Table 2) including 3 antibodies (CD45, CD31 and CK7) that allowed for manual gating of four distinct cell lineages: CD45+ leukocytes, CK7+ epithelial cells, CD31+ endothelial cells, and CD45-CK7-CD31- fibroblasts (Fig. 1b, gating strategy in Supplementary Fig. S7 and live cells counts in Supplementary Table 3). With this approach, we detected that the frequency of fibroblasts was 5-fold higher in fibrotic lungs (15\% in normal lungs compared to 80\% in fibrotic lungs), and leukocytes were 3-fold lower (60\% normal compared to 20\% in fibrotic lung). There was a mild but not significant decrease in epithelial cells and a negligible increase in endothelial cells (Fig. 1c). In addition to the increased abundance of
fibroblasts, we performed a principal component analysis (PCA) of the expression level of all the markers (except the lineage markers CD45, CK7, CD31, CD61 and CD235a) on fibroblasts and demonstrated that fibrotic lung fibroblasts from the 11 fibrotic lung patients clustered together and were distinct from lung fibroblasts derived from normal lungs (Fig. 1d), suggesting fibroblasts in fibrotic lungs are not only increased in percentage but also differed phenotypically from control-lung fibroblasts. Consistent with the PCA results, viSNE plots showed enrichment of a distinct fibrotic lung-specific fibroblast subpopulation (Fig. 1e). Mass cytometry also demonstrated co-activation of phospho JUN and AKT in 50% of fibroblasts in un-manipulated human fibrotic lungs (Fig. 1f). The fibrotic lung-specific fibroblast subpopulation expressed high levels of CD47 and podoplanin, whereas PDGFRα, calreticulin, and PD-L2 were moderately expressed (Supplementary Fig. S1a, b). As shown in Fig. 1g, >20% of the fibroblasts from fibrotic lungs expressed CD47 and a subset of ~10% co-expressed PD-L1. To assess the expression and distribution of these two immune checkpoint proteins in intact lung tissues, we performed immune staining of fibrotic and normal control lungs. We detected abundant co-expression of CD47 with FSP1, and PD-L1 with smooth muscle actin (SMA) in fibroblasts of fibrotic lung but not in normal lungs (Fig. 1h and Supplementary Fig. S1c showing the statistical analysis; Fig. S1d showing the H&E stains of the same fibrotic lung and normal lung) by immune fluorescent stains and 9-color multiplexed ion beam imaging (MIBI), a technology which allows concomitant staining of paraffin-embedded tissues with multiple antibodies and provides histologic resolution (Supplementary Fig. S1e).

We confirmed the upregulation of immune-checkpoint regulators at the gene-expression level with qPCR, where we detected increased *JUN*, *PD-L1* and *CD47* RNA expression in fibrotic over normal lungs (Fig. 1i). We also observed that secreted PD-L1 protein was increased in the bronchoalveolar lavages of fibrotic lungs but not normal lungs (Supplementary Fig. S1f). In conclusion, we found that fibroblasts in fibrotic lung are distinct from normal lung fibroblasts by both abundance and molecular phenotype, and a third of the fibroblasts upregulate either one of two immune checkpoint proteins, CD47 and PD-L1, with dual upregulation demonstrated in ~9%.
Macrophages and T cells derived from pulmonary fibrosis exhibit an immunosuppressive phenotype.

While many different inflammatory cell subsets have been shown to play a role in fibrotic lung, their individual contributions to pulmonary fibrosis progression are unclear. Here we used the unbiased, comprehensive, single-cell characterization mass cytometry method to deeply investigate millions of leukocyte types contained in fibrotic and normal lungs. Among all CD45+ leukocytes contained in the lungs, we found quantitative differences of B, NK and dendritic cells (Supplementary Fig. S2a) in fibrotic compared to normal lung controls. We observed no significant differences in the percentage of T-cells and macrophages (Fig. 2a, b).

For decades, investigations of the lung myeloid compartment have been mainly been limited to macrophages located within the airways, that is, alveolar macrophages which originate from fetal monocytes and are capable of self-renewal35,36. However, a number of recent reports have focused on the complexity of the myeloid cells present in the lung and provide evidence that interstitial macrophages are a heterogenous population comprising dendritic cells, tissue monocytes, and nonalveolar macrophages37. By using all of the markers in the CYTOF panel (except the lineage markers CD45, CD31, CK7, CD61, CD235a, cPARP, CD3, CD4, CD8, CD19, CD56 and CD68), we noticed macrophages from fibrotic lungs were phenotypically different from normal lungs as demonstrated by PCA (Fig. 2c). Consistently, viSNE plots also showed the distinct distribution of macrophages from fibrotic lung patients (Fig. 2d; black dotted circle). In addition, markers enriched in alveolar macrophages (e.g., HLADR, CD169 and CD206) (Fig. 2e), as well as indoleamine 2,3-dioxygenase (IDO) (a rate-limiting enzyme in the metabolism of tryptophan which plays a critical role in immune regulation) (Supplementary Fig. S2b, c) were down regulated in fibrotic lung macrophages. Indeed, the ratio of interstitial macrophages (HLA-DR+CD206+CD169−, IM)38 to alveolar macrophages (HLA-DR++CD206++CD169+, AM) was also significantly different between fibrotic and normal lungs (1.47 vs. 0.09, p=0.0079) (Fig. 2f, and Supplementary Fig. S2d).

Consistent with the high degree of phenotypic heterogeneity in macrophages (Supplementary Fig. S2d).
S2c), viSNE plots of manually gated alveolar and interstitial macrophages between fibrotic and normal lungs were also distinct, suggesting that development of unique AM and IM subtypes occurs in fibrotic lungs (Supplementary Fig. S2e). Meanwhile the macrophages in fibrotic lung tissues co-expressed PD-1 (Fig. 2g and Supplementary Fig. S2f showing the statistical analysis), a marker profile described for tumor-associated macrophages39. All these results suggest that the composition of macrophages changes dramatically during the fibrotic process.

Next we performed a thorough characterization of T cells. The percentage of T cells as well as CD4, CD8 and NKT cells did not differ between fibrotic and normal lungs (Supplementary Fig. S3a). However, an in depth analysis of T cells in pulmonary fibrosis demonstrated that specific subsets, such as naïve CD4 and naïve CD8 cells, were actually decreased (Fig. 3a, b) while no differences were detected for Th1, Th2 and Th17 T cells (Supplementary Fig. S3b). The most dramatic findings in fibrotic lung samples were the increased percentages of regulatory T cells (Treg) and PD-1+CD4+ T cells40 (Fig. 3c, d and Supplementary Fig. S3c), as well as the increased percentages of exhausted T cells (Fig. 3e, f), which are suggestive of an immunosuppressive microenvironment. We confirmed these findings with immune stains and, indeed, found a greater percentage of T cells in the fibrotic lungs expressing PD-1. (Fig. 3g and Supplementary Fig. S3d showing the statistical analysis). We conclude that suppressive leukocyte types predominate in pulmonary fibrosis lungs.

JUN transcriptionally controls profibrotic and immune-checkpoint-pathway genes.

Given the tight correlation between JUN activation and immune-checkpoint-protein expression in the fibroblasts at the single-cell level, we speculated that JUN might directly regulate CD47 and PD-L1 at the transcriptional level in fibrosis. Previous work by Vierbuchen et al.41,42 demonstrated that JUN, as part of the AP-1 (FOS/JUN) complex, can function as a pioneer transcription factor and acts as an enhancer selector to modulate the accessibility of DNA in fibroblasts. To that end, we conducted doxycycline (Dox)-inducible overexpression to investigate ectopic expression and CRISPR-editing to assay loss-of-function phenotypes with the goal of evaluating chromatin configuration in response to JUN expression using ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing).
This is a highly sensitive way to measure the chromatin accessibility of transposase with base-pair resolution genome-wide. We generated and performed ATAC-seq on primary fibrotic lung fibroblasts with (JUN-KO) or without (Control) genetic inactivation of JUN by CRISPR Cas9, followed by ATAC-seq and ChIP-seq on primary normal lung fibroblasts with (TetO-JUN Dox+, JUN-OE) or without (TetO-JUN Dox-) JUN overexpression. Hierarchical clustering of ATAC-seq signals revealed that the chromatin landscape of primary fibrotic lung fibroblasts was close to that of primary normal-lung fibroblasts with JUN activation; likewise, normal fibroblasts were clustered with fibrotic fibroblasts with JUN deactivation. Of the total open chromatin cis-regulatory elements across all samples, we found that the top differential peaks, enriched in pro-inflammatory genes such as CD47, CD274 (PD-L1), GLI1 and NFKB1, appeared to be regulated and downstream of JUN as their chromatin accessibilities decreased in JUN-KO fibrotic fibroblasts but increased in JUN-OE normal fibroblasts (Fig. 4a). JUN ChIP-seq data coupled with the ATAC data confirmed enrichment of bound JUN to the JUN promoter region (shaded in red), which correlates with the more accessible chromatin state in overexpressed JUN lung fibroblasts when compared to normal cells. This demonstrates that knockout of JUN decreases accessibility to its own promoter in a negative regulatory feedback fashion, while overexpression of JUN does the opposite. When we analyzed the DNA-bound JUN effects on the chromatin structure of CD47 or PD-L1, we noticed that JUN enrichment (by JUN ChIP-seq) occurs preferentially in a distal genomic region (shaded in green; previously shown to be a super-enhancer of CD47 in cancer43) for CD47 and in the first intronic genomic region (shaded in green; reported as a PD-L1 active enhancer44) for PD-L1, rather than in their corresponding promoters (shaded in red). The JUN enrichment observed in these two cases correlated with an increase in chromatin accessibility (detected by ATAC-seq) in lung-fibroblast cells compared to normal. This is particularly interesting as these changes are only present in our primary lung fibroblasts but not in any of the other previously published data on JUN ChIP-seq performed on cancer-cell lines such as A549, MCF7, H1-hESC, HepG2 or K562. These results suggest that the binding of JUN to specific CD47 and PD1 regions might modulate accessibility to DNA in regulatory regions specific to fibrotic
disease (Fig. 4b). We also compared the JUN KO against control and JUN-OE (TetO-JUN Dox+) against control (TetO-JUN Dox-) and found that chromatin peaks from promoters involved in the pro-fibrotic epithelial-mesenchymal transition, TGF-beta receptor signaling, and Stat3 signaling pathways were most significantly modulated with JUN modification (Supplementary Fig. S4a, b). In addition, to demonstrate the physiological relevance of these findings, we compared our ATAC-seq data with published gene expression profiling from fibrotic and normal lungs and found an overlap of 70 genes between the two datasets; among the most significant were genes encoding the profibrotic epithelial-mesenchymal transition pathway, indicating that the JUN pathway could be a driver of fibrotic progression in pulmonary fibrosis (Supplementary Fig. S4c).

To demonstrate JUN regulation of CD47 and PD-L1, we investigated their expression in fibroblasts following JUN modification after 4 days (Fig. 4c, d) and found that JUN-OE cells had higher levels of both RNA and protein expression. To confirm that changes in chromatin accessibility are pronounced in the enhancer region of CD47, we transduced fibrotic fibroblast cultures from patients with a construct containing the CD47-enhancer followed by a GFP reporter such that the enhancer activity can be monitored by GFP expression (Fig. 4e). To study whether the JUN pathway regulates CD47 through this enhancer, we serially measured GFP expression after JUN induction with a doxycycline-inducible system over a time course of 6 days and found that the CD47 enhancer was active with JUN expression. When we removed JUN from the culture by doxycycline withdrawal, we almost immediately lost CD47 enhancer activity (Fig. 4f and Supplementary Fig. S4d). On the other hand, GFP expression is further downregulated in JUN KO cells (Fig. 4g and Supplementary Fig. S4d). Together, these findings demonstrated JUN is one of the key factors that can remodel chromatin and increase DNA accessibility to regulate the expression of fibrotic genes.

Human fibrotic and mouse pro-fibrotic lung fibroblasts secrete IL-6 in a JUN-dependent manner and IL-6 signaling amplifies JUN-mediated pro-fibrotic effects via CD47.

To identify the cytokine pathways that cooperate with JUN, we profiled chemokines using a multiplex assay in the same bronchoalveolar lavage (BAL) human fibrotic lung samples that we analyzed by
mass cytometry. Remarkably, we discovered that IL-6 was among the most highly upregulated cytokines compared to BALs from normal donors, along with the PDGF-BB growth factor and CCL5/CXCL5, both critical factors shown to be involved in connective tissue remodeling\textsuperscript{46,47} (Fig. 5a). Additionally, we detected increased IL-6 and family members in our Jun-induced pulmonary fibrosis mouse lung washings (Fig. 5b). To determine which cells were responsible for IL-6 secretion, we quantified IL-6 in the supernatant of cultures of lung fibroblasts, whole bone marrow stroma, and bone-marrow-derived monocyte/macrophages. We detected Jun-dependent IL-6 secretion only in the explanted lung fibroblast and marrow stroma cultures but not in bone-marrow-derived monocytes/macrophage cultures which produced baseline IL-6 independent of Jun (Fig. 5c). These data indicated that IL-6 could be a critical downstream cytokine pathway involved in the Jun-mediated pro-fibrotic response.

We subsequently evaluated the dependency of IL-6 on JUN in primary human-lung fibroblast cultures in which \textit{JUN} was inducibly expressed or deleted with CRISPR Cas9. We found that promoter accessibility of \textit{IL-6} and \textit{IL-6R} and \textit{IL-6ST} depended on JUN since their accessibility (shaded in red\textsuperscript{48}) was lost with \textit{JUN} deletion; in contrast, JUN’s binding was highly enriched in the overexpressed-\textit{JUN} lung fibroblast cells, indicating increased \textit{IL-6} promoter accessibility in these cells (Supplementary Fig. S5a). IL-6 expression also correlated with JUN protein expression and, likewise, activation was lost with \textit{JUN} deletion (Fig. 5d). Blocking the IL-6 pathway has been shown to attenuate lung fibrosis in mice and human fibrotic lung; however, how IL-6 contributes to the fibrotic process itself is unclear\textsuperscript{34}. Our ELISA results demonstrate IL-6 concentrations of around 200 pg/ml in BAL of fibrotic lung patients (Supplementary Fig. S5b). Based on reported literature\textsuperscript{49} and our own quantitative IL-6 measurements in normal lung BAL, baseline levels of IL-6 in healthy men are around 2–10 pg/ml. Hence, we treated normal lung fibroblasts with 1, 10, 100 ng/ml IL-6 to investigate the influence of IL-6 on the \textit{CD47} enhancer. We found that increasing concentrations of IL-6 mimic JUN-mediated \textit{CD47} enhancer activity (Fig. 5e and Supplementary Fig. S5c) and increased CD47
protein expression (Fig. 5f). IL-6 addition to JUN KO fibroblasts had no effect on CD47 enhancer activity. Thus, IL-6 signaling cooperates with JUN to amplify JUN-mediated activation of the CD47 enhancer in a synergistic manner.

JUN induction in mouse lungs recapitulates the key molecular events found in human pulmonary fibrosis

In this paper, we have made the intriguing observations that two critical immune check point proteins—CD47 and PD-L1—are not only induced in a mouse model of lung fibrosis, but also in lung fibroblasts of human pulmonary fibrosis. These observations suggest that transcriptional programs active in fibrotic tissue try to dampen the immune response by blocking cytotoxic and phagocytic stimuli. Given these observations, we sought to determine whether blocking PD-1/PD-L1 would activate the immune system, both the innate and the adaptive, to eliminate overgrown fibroblasts in fibrotic lesions, and whether blocking different immune checkpoints simultaneously has additive anti-fibrotic effects and is well tolerated (Fig. 6). We previously established a Jun-inducible mouse model of lung fibrosis. Here, we compared our genetic model to the frequently used bleomycin-induced model of lung fibrosis and found that the fibrotic response in the chemical injury model was similar to our genetic lung-fibrosis model, i.e., similar distinct cell lineages [CD45+ leukocytes, EpCAM+ epithelial cells, CD31+ blood vessel endothelial cells and lineage negative fibroblasts (CD45-EpCAM-CD31-)] clustered together by X-shift clustering (Fig. 6a), and both resulted in activation of phospho JUN (Supplementary Fig. S6a).

Immune checkpoint inhibition and IL-6-signaling blockade stimulate T cells and macrophages to clear lung fibrosis in mice.

In the same study, we also found that the PD-L1 is upregulated in murine lung fibrosis in a subset of pro-fibrotic fibroblasts (in both the genetic and bleomycin chemical-injury models), which highly co-expressed CD47. In addition to the expansion of macrophages, we also detected an immunosuppressive microenvironment as was observed in human fibrotic lungs, including increased percentages of regulator T cells and exhausted T cells (Fig. 6b). We tested blockade of PD-1/PD-L1
(single/combined with anti-CD47 antibody) in the mouse model of lung fibrosis mediated by bleomycin in which we had initiated treatment at day 4 after fibrosis induction as a semi-therapeutic approach (Supplementary Fig. S6b). To assess and quantify the fibrotic response and the effects of immune checkpoint inhibition in vivo over time, we performed serial high-resolution CT imaging of the lung weekly and found a striking reduction of the fibrosis in the lung (highlighted by reduced radio densities), a result most notable in lungs of mice treated with a combined IL-6, PD-L1 and CD47 blockade (Fig. 6c, d). In addition, we analyzed the mice using mass cytometry coupled with histopathological and immune stains of the lungs two-weeks post-fibrosis initiation. Although there was increased PD-L1 co-expression with FSP1 in untreated mice with lung fibrosis, we found significantly decreased PD-L1+ CD47+ fibroblasts. FSP1 has been described as a good marker for profibrotic lung fibroblasts. This finding correlates with a decreased collagen content of lung sections of the mice which were treated with the triple combination of blocking antibodies against CD47 (Clone MIAP410) and IL-6 (Clone MP5-20F3), and an engineered non-antibody HAC protein which was reported to be an effective anti-PD-L1 blockade in the treatment of mouse tumor models. Similarly, B6.129S2-Il6tm1Kopf/J (IL-6 knockout) mice treated with anti-CD47 antibody and the PD-1 blocking reagent HAC similarly resolved their lung fibrosis confirming synergistic antifibrotic efficacy of IL-6 and immune checkpoint inhibition (Fig. 6e and Supplementary Fig. S6c-e). To evaluate the effects of immune checkpoint inhibition on innate immunity in fibrotic lung, we also used a humanized mouse model in which we have successfully engrafted primary human fibrotic lung fibroblasts in NOD-SCID gamma mice (NSG mice) underneath the kidney capsule. As key mediators of innate immunity, macrophages are the only remaining leukocytes in this NSG xenograft model and have been shown to interact with human PD-L1 via their PD-1. Based on luciferase detection of the human fibrotic fibroblast graft, we effectively resolved pathogenic pulmonary fibrosis by targeting PD-L1 with HAC protein. This outcome is confirmed by loss of GFP-positive grafted cells and lack of fibrosis by trichrome staining histology at the study endpoint (Supplementary Fig. S6f).
In summary, we demonstrated that immune checkpoint CD47, PD-L1 and IL-6 signaling was dramatically upregulated in a JUN-dependent fashion in pulmonary fibrosis patients and mouse lung-fibrosis models. We also showed that fibroblasts, macrophages and T cells in pro-fibrotic environments were all phenotypically different from the normal condition. Lastly, we established that blockage of JUN-controlled profibrotic and immune suppressive programs released immune suppression and hastened resolution of fibrosis in pulmonary fibrosis diseases (Fig. 7).

Discussion

Fibrosis is a reactive process initiated by the stimulation of fibroblasts by leukocytes. The progression of fibrosis is determined by a dynamic balance between anti-fibrotic and pro-fibrotic mediators within a microenvironment composed of diverse cellular subtypes. Therefore, it is essential to not only identify the multiple cell populations simultaneously, especially fibroblasts and leukocytes, but also monitor their activities for generating a better understanding the pathophysiology of pulmonary fibrosis. Single-cell mass cytometry offers a number of significant advantages for profiling fibrotic disorders and complements studies using single-cell transcriptomic approaches, such as the recently published single-cell RNA-seq study by Misharin and his colleagues. First, mass cytometry allows high throughput data collection in a single multiplexed experiment, thus enabling reliable comparisons of rare cell populations across different patients or conditions. Second, posttranslational modifications, which cannot be determined by transcriptomic analysis, are common mechanisms for regulating activities of proteins and can truly reflect their functional status inside cells. Finally, proteins but not mRNAs are the molecules which mediate most cross-talk between cells through ligand and receptor interactions on the cell surface. As demonstrated in this study, we profiled millions of cells from the same cohort in a single multiplexed experiment and showed the heterogeneity of fibroblasts in pulmonary fibrosis patients. The up-regulation of PD-L1 and CD47 in fibroblast subpopulations together with the immunosuppressive phenotype in T cells and macrophages suggested that the interactions between fibroblasts and leukocytes create a microenvironment in fibrotic lungs that...
restricts the removal of fibroblasts. The elevated activities of the JUN pathway in fibroblasts revealed by phospho-c-JUN-specific antibodies implicated the involvement of JUN pathways in the progression of pulmonary fibrosis. Indeed, by performing ATAC-seq, ChIP-seq and reporter assays, we demonstrated that the JUN pathway induces profibrotic and immunosuppressive gene expression through a mechanism which forms a positive feedback loop to increase CD47 expression via IL-6. Finally, the reduction of pulmonary fibrosis in mouse fibrosis models following blockade of IL-6, CD47 and PD-L1 validated the above mechanistic results, providing a potential therapeutic strategy to alleviate fibrosis in pulmonary fibrosis patients.

JUN is a transcription factor that coordinates the transcriptional regulation of genes that are essential for cellular growth and proliferation, such as the cell cycle, self-renewal, metabolism, and survival. Here we demonstrated that JUN expression in fibroblasts, contributes to fibrotic disease not only by directly increasing activity through cell-intrinsic, pro-fibrotic programs, but also by influencing the host’s overall immune response. We showed that JUN expression in fibroblasts increases IL-6 expression and secretion, which has direct effects on both the adaptive and innate immune systems. Furthermore, we discovered that JUN expression in fibroblasts upregulates the expression of the immune checkpoint genes CD274 (PD-L1) and CD47. Thus, the overexpression of JUN may be a general mechanism by which fibroblasts initiate the intrinsic fibrosis program and maintain the progression of fibrosis non-cell autonomously via interacting with the immune system. Interestingly, the dual effects of transcription factors as the key drivers of pathophysiology might be a general principle applicable to many disorders since a similar mechanism has been shown for MYC in cancer.

Clinically, CD47 and PD-L1 are of interest because excellent reagents have already been developed by multiple pharmaceutical companies to target both immune checkpoint molecules. Antibody therapies against both are currently being tested in clinical trials for cancer, and a blocking antibody against IL-6 is FDA approved to treat rheumatoid arthritis and acute cytokine release syndrome, a side effect of CAR-T cell therapy. Therefore, extensive safety information will become available from these cancer studies. However, so far the focus has been on treating cancer;
application of these immune therapies to other diseases has not been sufficiently been explored. Our work is the first to target several immune checkpoint and immune regulatory proteins in combination to achieve synergy in disrupting pro-fibrotic pathways in pre-clinical mouse models of fibrosis in a semi-therapeutic approach. In addition, our in vivo studies confirm our mechanistic studies, which are conceptually novel and demonstrate for the first time that JUN activation drives the expression of CD47 and PD-L1 in 30% of fibrotic lung fibroblasts and is mediated by IL-6 signaling. This finding will be of major importance to the design of future anti-fibrotic therapies based on immune regulatory proteins. Also, JUN inhibition (once systemic toxicity issues have been solved) may be by targeted delivery. Given the excitement and seemingly high success of targeting those two immune checkpoint pathways in cancer, clinical development for a different application like fibrosis will be greatly facilitated by piggybacking on the experience in cancer patients. While blocking antibodies against CD47 and IL-6 are relatively safe, clinical experience with PD-L1 inhibitors in cancer demonstrate severe pulmonary side effects potentially limiting their use and warranting caution in pulmonary fibrosis patients.

In conclusion, our data suggest that inhibition of each of these pathways (single/combined) in combination with standard of care therapy could potentially be used as a novel therapeutic approach for pulmonary fibrosis diseases. The consequences of our study are significant because our data represent a critical preclinical study that may ultimately be the basis for IND-enabling studies towards the goal of halting or even reversing the often-fatal course of pulmonary fibrosis diseases.

**Methods**

**Isolation of fibroblasts from human tissue.** Human fibroblasts were obtained from discarded fresh lung tissues from de-identified patients. The tissue was minced, filtered through 70 µm filters, and then centrifuged at 600 g for 5 min to remove non-homogenized pieces of tissue. The tissue homogenate was treated with ACK lysing buffer (Thermo Fisher) for 10–15 min, centrifuged at 600 g, washed twice in DMEM with 10% fetal bovine serum (Gibco), plated at a density of approximately
500,000 cells/cm² in DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin (Thermo Fisher Scientific) and Ciprofloxacin 10 μg/ml, Corning) and kept in an incubator at 37°C 95% O₂ / 5% CO₂. Media was changed after 24 h and cells were cultured until 80-90% confluent before each passage.

**Single Cell Mass Cytometry (CyTOF).** Samples were processed as described¹. Briefly the cell samples were fixed with 2% paraformaldehyde at room temperature for 20 min followed by two washes with PBS containing 0.5% BSA. Formaldehyde-fixed cell samples were incubated with metal-conjugated antibodies against surface markers for 1 h, washed once with PBS containing 0.5% BSA, permeabilized with methanol on ice for 15 min, washed twice with PBS containing 0.5% BSA and then incubated with metal-conjugated antibodies against intracellular molecules for 1 h. Cells were washed once with PBS containing 0.5% BSA, and then incubated at room temperature for 20 min with an iridium-containing DNA intercalator (Fluidigm) in PBS containing 2% paraformaldehyde. After intercalation/fixation, the cell samples were washed once with PBS containing 0.5% BSA and twice with water before measurement on a CyTOF mass cytometer (Fluidigm). Normalization for detector sensitivity was performed as previously described⁵⁴. After measurement and normalization, the individual files were analyzed by first gating out doublets, debris, and dead cells based on cell length, DNA content and cisplatin staining. ViSNE maps were generated with software tools available at https://www.cytobank.org by considering all surface markers.

**Immunostaining.** Tissue sections (4 μm thickness) for immunofluorescence staining were cut from tissue blocks of archival de-identified human biopsies using a microtome. The sections were baked at 65 °C for 20 min, deparaffinized in xylene and rehydrated via a graded ethanol series. The sections were then immersed in epitope retrieval buffer (10 mM sodium citrate, pH 6) and placed in a pressure cooker for 45 min. The sections were subsequently rinsed twice with dH₂O and once with wash buffer (TBS, 0.1% Tween, pH 7.2). Residual buffer was removed by gently touching the surface with a lint-free tissue before incubating with blocking buffer for 30 min. Blocking buffer was subsequently removed, and the sections were stained overnight at 4°C in a humidified chamber. The following morning, the sections were rinsed twice in wash buffer, a secondary antibody (Invitrogen, Carlsbad,
CA) was used for visualization of signal. Images of histological slides were obtained on a Leica Eclipse E400 microscope (Leica, Wetzlar, Germany) equipped with a SPOT RT color digital camera model 2.1.1 (Diagnostic Instruments, Sterling Heights, MI). For MIBI, slides were postfixed for 5 min (PBS, 2% glutaraldehyde), rinsed in dH₂O and stained with Hematoxylin for 10 s. At the end, the sections were dehydrated via a graded ethanol series and air dried using a vacuum desiccator for at least 24 h before imaging. MIBI imaging are performed by NanoSIMS 50L spectroscopy (Cameca, France) at Stanford Nano Shared Facilities (SNSF) and analyzed by using Image with Plugin OpenMIMS (NRIMS, http://www.nrims.hms.harvard.edu).

ELISA. Bronchoalveolar lavages were harvested from patient lungs immediately (within 5 minutes) after explant. Five ml were injected into the peripheral airspaces and at least 2 ml harvested for all specimens, which were subsequently snap frozen in liquid N₂. All specimens were surgical specimens and no post-mortem specimens were included. The expression of PD-L1 and IL-6 from bronchoalveolar lavages was quantitated following the protocols of ELISA kits: Human IL-6 Quantikine ELISA Kit and Human/Cynomolgus Monkey B7-H1/PD-L1 Quantikine ELISA Kit from R&D Systems.

Lentivirus preparation. 80–90% confluent 293T cells were transfected with 4 μg transfer plasmid (JUN tet-on overexpression plasmid, tetracycline-controllable transactivator plasmid, JUN CRISPR knock-out plasmid, TK control reporter plasmid, E7TK CD47 enhancer reporter plasmid and Luciferase-GFP plasmid), 2 μg pRRE Packing plasmid (GAG and Pol genes), 1 μg pRSV Packing plasmid (Rev gene), 1 μg pMD2.G enveloping plasmid and 24 μg PEI. The day after transfection, cell media was replaced, and cells were incubated for further 48 h, with media collection and replacement every 24 h twice. Cell media was centrifugated at 600 g for 10 min at 4 °C. Then, the supernatant was filtered through a 0.22 μm strainer, ultra-centrifuged at 25,000g for 2h, aliquoted and flash frozen.

CRISPR-mediated genome engineering. Following literature protocols⁵⁵,⁵⁶, the sequences of 2 site-specific guide RNAs (sgRNAs) that target exon1 of the JUN gene were selected using the CRISPR
Design Tool 43. Oligonucleotides with these sequences were cloned into the lentiCRISPRv2 vector (Addgene, Cambridge, MA) which uses Puromycin selection to enrich for cells with \textit{JUN} knock out.

The sgRNA sequences:

\textbf{\textit{JUN} sgRNA\_1 F} \hspace{1cm} \textcolor{blue}{CACCCTGAACCTGGCCGACCCAGTG} \\
\textbf{\textit{JUN} sgRNA\_1 R} \hspace{1cm} \textcolor{blue}{AAACCACCTGGGTCCGACCCAGGTTCAC} \\
\textbf{\textit{JUN} sgRNA\_2 F} \hspace{1cm} \textcolor{blue}{CACCCTCGTGAGAGCGGACCTTA} \\
\textbf{\textit{JUN} sgRNA\_2 R} \hspace{1cm} \textcolor{blue}{AAACTAAGGTGCCTCTCGACGGGC} \\

\textbf{Doxycycline (DOX) Inducible \textit{JUN} overexpression.} To generate the \textit{JUN} tet-on overexpression plasmid, we cloned \textit{JUN} cDNA into IRES-Hygro-TetO-FUW vector (Addgene, Cambridge, MA). To generate doxycycline (Dox) inducible \textit{JUN} overexpression, the tetracycline controllable transactivator (rtTA) lentivirus was infected with the \textit{JUN} tet-on overexpression plasmid. Dox (2 mg/ml) was applied during the infection to turn on \textit{JUN} overexpression. Hygromycin selection was started on the second day for 2 days.

\textbf{ATAC-seq and ChIP-seq library preparation and sequencing library preparation.} The primary fibrotic lung fibroblasts were infected with \textit{JUN} knock-out lentiviruses, followed by 4 days of puromycin selection; meanwhile the normal lung fibroblasts were infected with \textit{JUN} overexpression lentiviruses, followed with 2 days hygromycin selection. As described in detail in the published protocol\textsuperscript{57} a transposition reaction was initiated in each sample containing 50,000 nuclei as assessed by counting. Subsequently a DNA library was prepared using a Nextera DNA Library Preparation Kit (Illumina) and sequenced on the Illumina Nextseq 500 platform with 75-bp \times 2 paired-end reads. ChIPs and their respective inputs were generated as previously described\textsuperscript{43}. The libraries were prepared by using a TruSeq ChIP sample preparation kit (Illumina) and sequenced by Nextseq500 pair end sequencing (75bp).

\textbf{Deep sequencing data analysis.} ATAC-seq and ChIP-seq data analysis used the Kundaje lab pipeline with the following tools and versions: Cutadapt v 1.9.1, Picard v1.126, Bowtie2 v2.2.8,
MACS2 v2.1.0.20150731, and Bedtools v 2.26. First, Nextera adaptor sequences were trimmed from the reads by using Cutadapt program v 1.9.1. These reads were aligned to human genome hg38 using Bowtie2. The standard default settings were modified to allow mapped paired-end fragments up to 2 kb. Only the reads with mapping quality greater than 30 were kept, and the duplicated reads were removed using Picard tools v1.126. The reads from mitochondria were also removed, then we converted PE BAM to tagAlign (BED 3+3 format) using Bedtools v 2.26 functions. Differential expression analysis were done by DESeq2 (http://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html). Differential peaks had a p-value less than 0.01 and absolute log2 fold change above 1.

**Flow Cytometry.** The analysis of IL-6 or surface molecules (CD47 and PD-1) was performed using monoclonal antibodies listed in the Supplementary Table 2. Data were acquired by LSRII or LSRFortessa flow cytometers and analyzed using FlowJo software or Cytobank.

**Protein expression and purification.** Proteins blocking human or mouse PD-L1, (HACV or HACmb respectively) were produced as described previously58.

**Bleomycin-induced mouse model and JUN-induced lung fibrosis mouse model.** For bleomycin administration, mice were anaesthetized with isoflurane followed by intratracheal instillation of bleomycin (4 U/kg per body weight) in 100 μl PBS as previously described59. As published previously1, the reverse tetracycline transactivator (rtTA) ubiquitously inducible Jun was expressed using the Rosa26 promoter, and the inducible cassette was targeted downstream of Col1a1 promoter. After crossing, genotyping of the inducible JUN mice was performed using primers for the transgene JUN and Rosa26. JUN under the Col1a1 promoter was induced by adding doxycycline (2 mg/ml) (Millipore Sigma) to the drinking water.

**In Vivo Antibody Blockade.** For CD47 antibody blockade experiments, mice were injected intraperitoneally (IP) with a dose of 500 μg CD47 antibody (Clone MIAP410, Bioxcell) diluted in 100 μl of PBS on day 4. The same dose was then given every other day up to two weeks. For PD-L1 blockade experiments, HAC protein (250 μg, IP) was given daily for the entire treatment period. For
IL-6 antibody blockade experiments, mice were injected intraperitoneally with 20 mg/kg dose of an anti-IL-6 monoclonal (Clone MP5-20F3, Bioxcell) antibodies twice a week for 2 weeks.

**CT scan.** Mice were anesthetized, and CT Scans were performed using a Bruker Skycan 1276 (Bruker, Belgium). CT scans were then analyzed with Bruker Skycan tools. To determine dense areas within the lungs, binary pictures were created using the heart as the cutoff value to split the tissue in dark (having at the least the same density as the heart) and white areas. Representative total lung and dense areas were measured in the upper and middle field. Finally, the fraction of the dense areas within the total lung areas was calculated.

**Kidney capsule transplantation.** After mice had been anesthetized, the areas over the right and/or left flank were shaved and disinfected. Thereafter, a flank cut was made. Subcutaneous tissues were bluntly removed and an incision into the abdominal cavity was made. The kidney was luxated out of the abdominal cavity and a slight incision into the renal capsule was made. The renal capsule was bluntly detached from the renal tissue and $2 \times 10^5$ cells suspended in 10 μl of matrigel were injected under the kidney capsule. The kidney was pushed back into the abdominal cavity. The abdominal cavity and skin were closed using sutures.

**Luciferase-based optimal imaging (BLI).** 100 μl of luciferin substrate was intraperitoneally injected. Fifteen minutes later, optical imaging was performed using a Lago optical imaging system (Spectral imaging instruments, AZ, USA). Analysis was done with the Aura Software from the same manufacturer.

**Statistics.** Statistical analyses were performed using Prism software (GraphPad Software). Statistical significance was determined by the unpaired Student’s t test for comparisons between two groups and one-way ANOVA for multigroup comparisons (n.s. non significant; $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). In statistical graphs, points indicate individual samples, and results represent the mean ± SD unless indicated otherwise.
Data availability. Raw ATAC-seq and ChIP-seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession code GSE114844. JUN ChIP-seq of HepG2 (GSM935364), MCF-7 (GSE91550), H1-hESC (GSM935614), A549 (GSE92221) and K562 (GSM1003609), Histone ChIP-seq data of H3K4me3 (GSM733723), H3K27ac (GSM733646), H3K9me3 (GSM1003531) and H3K27me3 (GSM733764) and RNA-seq data of fibrotic lungs (GSE52463) are from the public GEO database.

Study approval. De-identified patient specimens in paraffin and discarded fresh patient tissues were used for our studies as approved in IRB-39881 and -18891. We received primary lung tissues exclusively from patients with end-stage pulmonary fibrosis undergoing transplantation. Therefore, our cohort of patients represents severely fibrotic lung disease. It has been challenging to receive normal control lung tissues. While we have received lung tissues from normal lung resections from tumor resections from Stanford tissue bank as well as lungs from rapid autopsies, it appeared that the only normal lung tissues harvested during surgery by the tissue bank were of sufficient viability to include in our CyTOF studies; while other cell-type fractions appeared representative we noted a bias towards less endothelial cells in the normal biopsies due to the relatively small amounts of lung tissue we received from the tissue bank. Mice were maintained in Stanford University Laboratory Animal Facility in accordance with Stanford Animal Care and Use Committee and National Institutes of Health guidelines (SU-APLAC 30912).

Author contributions
L.C., S-Y.C. and G.W. planned all experiments, analyzed the data, G.W. wrote the paper, L.C., S-Y.C., T.L. and P.D. performed all experiments; J.L., P.B., and G.N. contributed new reagents/analytic tools.

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Figures

Figure 1

a) Systems-level analysis

- Cell populations
  - (Mass Spectrometry, Immunofluorescence and Q-PCR)
- Bronchoalveolar lavage proteins
  - (Luminex Multiplex Assays and ELISA)
- Cell functions
  - (Mass Spectrometry, ATAC-seq, MIBI Reporter assay and Flow Cytometry)
- Immunotherapy
  - (anti-IL-6, anti-CD47 and anti-PD-L1 treatment)

b) Whole Lung

- CD31+ Endothelial cells
- Lin- Fibroblast
- CK7+ Epithelial cells
- CD45+ Leukocytes

Percentage

- Fibrotic Lung
- Normal Lung

Whole Lung

- Fibroblasts
- Leukocytes
- Epithelium
- Endothelium

Fibroblasts (PCA)

- PC1 (30.7%)
- PC2 (18.9%)

Fibroblast Abundance

- RNASE2
- hT3E1

Fibrotic Lung

- PD-L1
- CD47

Normal Lung

- PD-L1
- CD47

h) CD47 FSP1 MERGE

i) JUN (QPCR) PD-L1 (QPCR) CD47 (QPCR)

- RNA Expression (Fold Change)
- Fold Change

Fibrotic fibroblast Normal fibroblast

- Fibrotic fibroblast Normal fibroblast

Fibrotic fibroblast Normal fibroblast
Figure 1. Systems-level analysis of pulmonary fibrosis patients demonstrating a key role for immune-checkpoint proteins PD-L1 and CD47 in fibrotic lung fibroblasts as well as JUN. (a) Outline of our “-omics” approach in human fibrotic lung integrating proteomics, secretomics, and genomics technology platforms to study the contribution of leukocytes and pathologic fibroblasts and to identify new therapeutic targets. (b) Single-cell, force-directed layout of fibrotic lung tissues. Shaded regions indicate the location of manually gated cell populations: green-shaded area represents leukocytes (CD45+), pink area represents epithelial cells (CK7+), blue area represents endothelial cells (CD31+) and grey dotted circle highlights the fibroblasts (CD45-CK7-CD31-). (c) Frequencies of cell populations in the lung detected by mass cytometry (CyTOF). Data are displayed as mean ± SD of 11 fibrotic and 3 normal control lung samples. (d) Principal component analysis (PCA) computed on mass cytometry data sets from fibroblast clusters from 11 individual pulmonary fibrosis patients (PF) and 3 normal donors (NC) demonstrating that fibrotic and normal fibroblasts were distinct from each other. (e) ViSNE maps of fibroblast mass cytometry data demonstrating that the abundance of fibroblasts differed; in normal controls the lung fibroblasts appeared heterogeneous while the fibroblasts clustered tightly together in fibrotic lungs (Blue: highlighted by the black dotted circle). The data demonstrate a representative example per group and each point in the viSNE map represents an individual cell. (f) ViSNE analysis of mass cytometry data of fibrotic lung (blue dots), normal lung (orange dots) and normal peripheral blood mononuclear cells (PBMCs, green dots) revealed increased activation of the JUN and AKT pathways in fibrotic lung fibroblasts. Schematic diagram of the location of the indicated cell types on the viSNE map are based on the expression of lineage-specific markers: epithelial cells (Epi), natural killer cells (NK), plasmacytoid dendritic cells (pDC), endothelial cells (EC), and macrophages (Mac). Red indicates high and blue low protein expression. (g) Representative mass cytometry plots of the pro-fibrotic fibroblast population in fibrotic lung compared with normal lung. (h) Immune fluorescent stains confirmed increased CD47 and PD-L1 co-expression in lung fibroblasts from fibrotic lungs compared to normal controls (activated fibroblasts expressing FSP1+ Collagen1+ and SMA+). The arrow indicates the blood vessel. (Scale
bars, 100 μm). (i) RNA expression analysis of JUN, PD-L1 and CD47 in fibrotic and normal lung fibroblasts as detected by QPCR. Data are expressed as mean ± SD of 5 fibrotic fibroblasts and 3 normal fibroblasts and are representative of at least three experiments. Data were analyzed by two-tailed unpaired t-test, * P < 0.05. See Supplementary Table 4 for statistical details.

**Figure 2.** Immune suppressive subsets of macrophages predominate in lung fibrosis. (a) Main cluster frequencies of CD45+ leukocytes (T cells, macrophages, B cells, NK cells, dendritic cells and other inflammatory cells such as neutrophils/eosinophils/plasma cells) contained in the lungs of pulmonary fibrosis patients and normal controls were quantified by mass cytometry. Data are expressed as mean ± SD of 11 fibrotic and 3 normal lung samples. (b) Computational analysis of mass cytometry data of leukocytes derived from fibrotic lungs with a single-cell, force-directed algorithm demonstrated that the different inflammatory subsets segregated as indicated on the map: CD4+ T cells (blue), CD8+ T cells (purple), macrophages (green), B cells (orange), NK cells (yellow) and the dendritic cell subset (white). (c) Principal component analysis (PCA) of manually gated...
macrophages (CD45+CD68+nonB nonT, nonNK Live cells) indicating that macrophages derived from the pulmonary fibrosis lung (PF) clusters are distinct from those in normal lungs (NC). (d) A refined viSNE analysis of mass cytometry data demonstrating that macrophages derived from normal lungs (orange) have a distinct profile from fibrotic lungs (blue: black dotted circle). (e) ViSNE analysis of macrophages isolated from normal lungs and fibrotic lungs demonstrating decreased activation of HLA-DR, CD169 and CD206 expression in fibrotic lungs relative to controls. Each point represents a single cell, and the samples are color coded as indicated: blue colors represent low expression and yellow to red represent high protein expression. (f) The corresponding ratio of interstitial macrophages (HLA-DR+CD206+CD169−, IM) versus alveolar macrophages (HLA-DR++CD206++CD169+, AM) is displayed with mean ± SD of 11 fibrotic and 3 normal lung samples and analyzed by two-tailed unpaired t-test, ** P < 0.01. (g) Representative images of immune fluorescent stains highlighted increased PD-1 expression in macrophages from fibrotic lung tissues (Scale bars, 100 μm). See Supplementary Table 4 for statistical details.

Figure 3
Figure 3. T cells present in fibrotic lungs mitigate a suppressive immune response in lung fibrosis. (a, b) Representative CyTOF plots and quantification of CD4+ (top) and CD8+ (bottom) naïve T cells (CCR7+ CD45RA+) demonstrating decreased naïve T cells in fibrotic lungs. Data represent mean ± SD of 11 fibrotic and 3 normal samples and are analyzed by two-tailed unpaired t-test, *** $P < 0.001$; **** $P < 0.0001$. (c, d) Representative CyTOF analysis showing increased frequency of regulatory CD4 T cells (Treg: CD4+Foxp3+CD25+) in fibrotic lungs. Data represent mean ± SD of 11 fibrotic and 3 normal samples and are analyzed by two-tailed unpaired t-test, *** $P < 0.001$. (e, f) Representative CyTOF plots and quantitative analysis indicating increased percentage of exhausted T cells (Tex: CD8+PD-1+TIM3+) in fibrotic lungs. Data represent mean ± SD of 11 fibrotic and 3 normal samples and are analyzed by two-tailed unpaired t-test, * $P < 0.05$. (g) Representative images of immune fluorescent stains for PD-1 on T cells (CD3+) highlighting increased percentage of PD-1+ T cells in fibrotic lung samples (Scale bars, 100 μm). See Supplementary Table 4 for statistical details.
Figure 4

(a) Color Key and Histogram

(b) ATAC

(c) RNA Expression (QPCR)

(d) Protein Expression (Flow)

(e) Control reporter vector

(f) JUN time course treatment (CD47 Enhancer)

(g) JUN overexpression vs knockout (CD47 Enhancer)
Figure 4. Promoter accessibility of CD274 (PD-L1) and CD47 in fibrotic lung fibroblasts depends on JUN and directly appears to regulate the enhancer of CD47. (a) Heatmap demonstrating dynamic chromatin changes in fibrotic lung fibroblasts with (JUN-KO) or without (Control) JUN deletion and normal lung fibroblasts with (TetO-JUN Dox+) or without (TetO-JUN Dox-) JUN activation. (b) Representative genome browser tracks comparing ATAC-seq signal in fibrotic lung fibroblasts (with (JUN-KO) or without (Control) JUN-knockout) and also ChIP-seq signal in normal lung fibroblasts (with (TetO-JUN Dox+) or without (TetO-JUN Dox-) JUN overexpression) with A549, MCF7, h1-hESC, HepG2 and K562 from published data at JUN, CD47 and CD274 loci. The red boxes highlight ATAC-seq and ChIP-seq peaks in the promoter sites of JUN, CD47 and CD274 (and enhancer is shown in green). We also compared our peaks with H3K4me3 or H3K27Ac (=histone mark for open chromatin), H3K9me3 or H3K27me3 (=histone mark for closed chromatin), ChIP-seq data generated from normal human lung fibroblasts is from published data, which highlighted the same areas respectively. (c) Gene expression changes in primary lung fibroblasts from JUN knockout (KO) compared to overexpression (OE). QPCR values were normalized to the value in JUN KO. Four experimental repeats. Ratio paired t test, ** P < 0.01; **** P < 0.0001. (d) Representative flow cytometry histograms showing reduced expression of pJUN, PD-L1 and CD47 after JUN overexpression (OE) or KO. Yellow plot: JUN overexpression; Black plot: JUN knockout. (e) Vector maps of the control and CD47 enhancer constructs used to engineer reporter cell lines. (f, g) CD47 enhancer reporter assays demonstrating doxycycline-induced JUN expression initiated CD47 enhancer expression which disappeared when JUN expression was turned off (f) or JUN was knocked out (g). Data are expressed as mean ± SD, Ordinary one-way ANOVA (Tukey’s multiple comparisons test), n.s., non-significant; * P < 0.05; *** P < 0.001; **** P < 0.0001. See Supplementary Table 4 for statistical details.
Figure 5. IL-6 mediates the pro-fibrotic response of JUN. (a) The secreted proteins in the lung bronchoalveolar lavage (BAL) of fibrotic lung patients were quantified by Luminex assay, showing IL-6 as the highest expressed cytokine across all fibrotic patient BAL samples. Data were normalized by protein levels of the BAL of normal lung and presented as mean ± SD. (b) Cytokines and chemokines in the fibrotic mouse BAL after Jun induction were quantified by Luminex assay. IL-6 was consistently among the most highly expressed cytokines in Jun-induced mouse fibrotic lungs indicative of IL-6-JAK-STAT pathway activation. Data were normalized by normal lung expression and presented as mean ± SD. (c) The cytokines/chemokines released from Jun-induced, lung-fibrotic, mouse-derived whole bone marrow, fibroblasts and monocytes/macrophages in the medium after 48h of Dox-initiated Jun induction were quantified by Luminex assay, demonstrating that whole bone marrow and fibroblasts are secreting increased IL-6 in response to Jun. Data are presented as mean ± SD. (d) Increased IL-6 expression levels were detected by QPCR and flow cytometry in primary lung fibroblasts with JUN knock-out (KO) or overexpression (OE). Four experimental repeats. Ratio paired t test, *** \( P < 0.001 \). (e, f) IL-6 increased CD47 enhancer activity at concentrations as low as 1 ng/ml (e) and protein expression at 10 ng/ml (f) in a dose-dependent fashion. Data are expressed as mean ± SD, Ordinary one-way ANOVA with multiple comparisons test, n.s., non-significant; ** \( P < 0.01 \); *** \( P < 0.001 \); **** \( P < 0.0001 \). See Supplementary Table 4 for statistical details.
Figure 6. Inhibition of immune checkpoints together with IL-6 resolves lung fibrosis. (a) Whole-lung scaffold map for bleomycin-induced lung fibrosis in mice. Each node represents unsupervised cell clusters. (b) Representative mass cytometry plot demonstrating increased expression of immune checkpoint proteins CD47 and PD-L1 in fibroblasts and an expansion of CD11b+F4/80+ macrophages, regulator T cells (CD3+CD4+CD25+FOXP3+) and exhausted T cells (CD3+CD8+PD-1+TIM3+) in mouse model after fibrosis induction with bleomycin for 2 weeks. (c, d) Representative images of Micro CT scans of wildtype and B6.129S2-Il6<sup>tm1Kopf</sup>/J (IL-6KO) mice highlighting increased fibrosis in the lung after fibrosis induction (wildtype and IL-6KO mice) and much improved fibrosis after treatment with HAC (anti-PD-L1) alone or combined with a blocking antibody against CD47 or/and IL-6. Data are expressed as mean ± SD of 5 animals and analyzed by using one-way ANOVA.
for multiple comparisons test. n.s., non-significant; * $P < 0.05$; **** $P < 0.0001$. (e) Trichrome of lung sections of control mice, mice after fibrosis induction with bleomycin (wildtype and IL-6KO mice) and mice after treatment with blocking antibodies against IL-6 and CD47 and HAC (the blocking reagent against PD-L1) demonstrating dramatically improved fibrosis (significantly decreased blue stained areas on Masson’s trichrome stain which correspond to cross-linked collagen) and diminished PD-L1 expression in FSP1+ fibroblasts after treatment. Scale bar, 100 μm. See Supplementary Table 4 for statistical details.

Figure 7. Schematic diagram of the proposed mechanisms of fibrosis clearance. Left: In fibrotic lung, we find persistent myofibroblast activation in fibrotic plaques and JUN upregulation. JUN expression in fibrosis-associated fibroblasts (FAFs) appears to directly control the promoters and enhancers of CD47 and CD274 (PD-L1). The direct consequence is increased expression of these immune checkpoint proteins in fibroblasts and dormant macrophages which do not phagocytose, but continue to release chronic inflammatory cytokines. JUN also directly regulates IL-6 at the chromatin level. The increased expression and secretion of this potent cytokine leads to a suppressive adaptive
immune response—chiefly T cell exhaustion and upregulation of regulatory T cells. Right: Disrupting
the suppression of the innate and adaptive immunity with CD47 and PD-L1 inhibitors as well as the
proinflammatory IL-6 cytokine pathway stimulated phagocytic removal of profibrotic fibroblasts and T-
cell activation leading to clearance of the fibrosis in the lung.

Supplementary data

Supplementary Figures
**Figure S1**

(a) ViSNE map of concatenated fibroblasts (CD45-CD31-CK7- population) from fibrotic lung (black dotted circle) and normal lung demonstrating increased expression of PDGFRα, podoplanin, CD47 and PD-L2 but not calreticulin in subsets of fibroblasts in fibrotic lungs. (b) Representative CyTOF plots of PD-L2 and calreticulin protein in fibroblasts from fibrotic and normal lungs indicating increased PD-L2 but no difference in calreticulin expression. (c) Quantitation of CD47 and PD-L1 expression in BAL (ELISA).
immune stains in fibrotic and normal lung biopsies. Data are expressed as mean ± SD and analyzed by two-tailed unpaired \( t \)-test, ** \( P < 0.01 \); **** \( P < 0.0001 \). The immune stains were evaluated by a blinded pathologist, in addition to image J software. (d) A representative haematoxylin and eosin staining of fibrotic and normal lung tissue. The inserted black frames highlight the fibrotic and normal areas. Scale bar, 100 \( \mu \)m. (e) Multiplexed ion beam imaging (MIBI) and relevant quantitation demonstrated the co-expression of JUN and FOS with CD47 in fibroblasts in fibrotic plaques in lungs of idiopathic pulmonary fibrosis patients. Representative MIBI analysis of lung biopsy sections from 5 patients with idiopathic pulmonary fibrosis were stained with metal-conjugated antibodies. In total, 10 different markers (JUN, JUNB, JUND, FRA1, FRA2, FOS, FOSB, COLLAGEN1, CD47 and Hematoxylin) were analyzed. Eight fields of view were acquired with ten repeat scans over a single area. Experiments were run multiple times, representative examples and related analyses are shown as mean ± SD. Scale bar, 100 \( \mu \)m. (f) ELISA detected increased levels of secreted PD-L1 in fibrotic lung BAL compared to normal lungs. Data are expressed as mean ± SD of 5 fibrotic and 3 normal samples. Data were analyzed by two-tailed unpaired \( t \)-test, * \( P < 0.05 \). See Supplementary Table 4 for statistical details.
Supplementary Figure S2 (Refers to Figure 2).
We analyzed dendritic cells in fibrotic and normal lungs with mass cytometry and found increased percentages of myeloid dendritic cells (mDC: CD45+ nonB nonT nonNK nonmacrophage CD11c+CD123-) in fibrotic lung but no difference for plasmacytoid dendritic cells (pDC: CD45+ nonB nonT nonNK nonmacrophage CD11c-CD123+). (b) IDO protein expression in macrophages from fibrotic lungs is decreased compared to macrophages from normal control lungs. Raw values of means of CyTOF data are displayed on a per-patient basis with mean ± SD of 11 fibrotic and 3 normal samples and analyzed by two-tailed unpaired t-test, * P < 0.05. (c) The viSNE maps colored by intensity of expression (red is high, and blue is low) demonstrate the expression of IDO, ARG1, CD47, CD16, CD163 and CD11c in macrophages derived from fibrotic lungs which clustered spatially within the black circled area. (d) Representative histogram of mass cytometry data demonstrates decreased alveolar macrophages (AM) but increased interstitial macrophages (IM) in human fibrotic lungs. (e) Individual viSNE analysis of AM and IM from fibrotic lung (blue) and normal lungs (orange) suggested the immunophenotypes of AM and IM in the fibrotic tissues are clearly different from those in the normal lungs. Macrophages derived from fibrotic lungs are highlighted by the dotted black circles. (f) Quantitation of PD-1+ expression on macrophages (CD68+) in fibrotic and normal lung biopsies. Data are expressed as mean ± SD and analyzed by unpaired t test with Welch’s correction (Two-tailed), ** P < 0.01. The immune stains were evaluated by a blinded pathologist, in addition to image J software. See Supplementary Table 4 for statistical details.
**Supplementary Figure S3 (Refers to Figure 3).**

(a) We quantified the frequencies of T cells and found no significant differences in total CD4 and CD3 T cells, but decreased percentages of NKT cells in individual samples (plotted as a fraction of total T cells and displayed as mean ± SD). (b) Representative plots of mass cytometry data showing no significant difference of CD4 T-cell subsets, polarized Th1 (CD3+CD4+TBET+), Th2 (CD3+CD4+GATA3) and Th17 (CD3+CD4+RORgc) in fibrotic and normal lungs. (c) Percentage of PD-1+ expressing CD4+ T cells are displayed with mean ± SD of 11 fibrotic and 3 normal lung samples. Data are expressed as mean ± SD and analyzed by two-way unpaired t-test, **** P < 0.0001. (d) Quantitation of PD-1+ expression on T cells (CD3+) in fibrotic and normal lung biopsies. Data are expressed as mean ± SD and analyzed by unpaired t test with Welch's correction (Two-tailed), *** P < 0.001. The immune stains were evaluated by a blinded pathologist, in addition to image J software. See **Supplementary Table 4** for statistical details.
Supplementary Figure S4 (Refers to Figure 4).

Figure S4

a) Fibrotic lung fibroblast knock-out vs untreated

- Total peaks number: 243389
- 6.29% Down-regulated
- 6.09% Up-regulated

b) Normal lung fibroblast over-expression vs untreated

- Total peaks number: 326295
- 5.23% Down-regulated
- 6.49% Up-regulated

c) Promoter Down vs RNAseq Up

- 3700 (85.6%)
- 70 (1.6%)
- 552 (12.8%)

Gene Set Enrichment Analysis (GSEA)

- Epithelial-Mesenchymal Transition
- Coagulation
- UV Response Down
- Inflammatory Response
- Estrogen Response Early
- KRAS Signaling Up
- TNFA Signaling via NFkB

- Hallmark Gene Set

- GSEA analysis for JUN-OE and JUN-KO

Supplementary Figure S4 (Refers to Figure 4).
(a, b) Quantitative comparative analysis of ATAC-seq peaks obtained from fibrotic lung fibroblasts with and without *JUN* deletion as well as normal lung fibroblasts with or without *JUN* overexpression. The top ten significant pathways which were associated with down regulation (labeled as Promoter Down in red) or up regulation (labeled as Promoter Up in blue) of the promoters were shown. (c) Venn Diagram generated by comparing downregulated promoters in fibrotic lung fibroblasts after *JUN* deletion with published RNA-seq data of bulk fibrotic lung samples demonstrating that 1.6% or 70 of the genes which overlapped between these two distinct data sets encoded profibrotic pathways (red) and pathways which encoded T-cell exhaustion (green). (d) Reporter assays for the *CD47* enhancer demonstrating continuously increasing activation of the *CD47* enhancer (E7TK) reflected by increased *EGFP* expression with increased *JUN* expression (*JUN-OE*) while the *CD47* enhancer activity decreased with doxycycline removal (turns *JUN* off) in a timely dependent manner and *JUN* deletion with CRISPR-Cas9 knock-out (*JUN-KO*) abolished the enhancer activity. Meanwhile the control TK vector showing no differences with *JUN* modification. Scale bar, 100 μm. See Supplementary Table 4 for statistical details.
Supplementary Figure S5 (Refers to Figure 5).
(a) Schematic maps showing that the promoter sites (highlighted in red) for *IL-6*, *IL-6R*, and *IL-6ST* depended on *JUN* expression in normal lung fibroblasts with (TetO-*JUN* Dox+) or without (TetO-*JUN* Dox-) *JUN* overexpression and fibrotic lung fibroblasts with (*JUN-KO*) or without (Control) *JUN* knockout with CRISPR-Cas9 but not in other cell lines like A549, MCF7, h1-hESC, HepG2 and K562. We also compared our data to publicly available H3K4me3 or H3K27Ac (=histone mark for open chromatin), H3K9me3 or H3K27me3 (=histone mark for closed chromatin) ChIP-seq data generated from normal human lung fibroblast from published data to confirm the regions of open chromatin for the *IL-6* family members. (b) *IL-6* expression in the bronchoalveolar lavages (BAL) of fibrotic and normal lungs were measured by ELISA showing dramatically increased secreted IL-6 protein. Data are expressed as min to max of 5 fibrotic and 3 normal samples. Data were analyzed by unpaired t test with Welch’s correction (Two-tailed), *** $P < 0.001$. (c) *CD47* constituent enhancer-driven EGFP reporter (E7TK) expression was activated and increased in lung fibroblast cells treated with IL-6 in a dose dependent manner. Control cells were transduced with the lentiviral cassette containing the thymidine kinase (TK) minimal promoter only. Scale bar, 100 μm. See Supplementary Table 4 for statistical details.
Figure S6

a) Fibroblasts

Mass Cytometry

Fibroblasts

bleomycin treated model
PBS treated control

Mice with JUN induction
Mice without JUN induction

b) Days

0
Beginning HAC/aCD47/aIL-6
Administration

4
( or PBS) treatment

14
Micro CT
Animal euthanasia
Post-mortal analysis

WT

b) Tbleomycin

aCD47/HAC

aCD47/HAC

aCD47

HAC

PBS

IL6KO

b) Tbleomycin

aCD47

aCD47/HAC

aCD47/HAC

aCD47

HAC

PBS

c) H&E

CD47

IL6KO

PD-L1

DSP-1

IL6KO PBS

WT

b) Tbleomycin

aCD47

aCD47/HAC

aCD47/HAC

aCD47

HAC

PBS

e) PD-L1

% fibroblast

n.s.

n.s.

n.s.

n.s.

CD47

% fibroblast

n.s.

n.s.

n.s.

n.s.

Collagen

% fibrosis

f) Days

0
Kidney Capsule Transplantation

Confirmation engraftment

Beginning HAC (or PBS) treatment

7 days

14
BLI

21
BLI

Animal euthanasia

Post-mortal analysis

Pre-treatment

PBS HAC

Trichrome

GFP

HAC

IPF
Supplementary Figure S6 (Refers to Figure 6).

(a) Histogram plots of mass cytometry data of phosphor p-JUN expression in lung fibroblasts comparing two different mouse models of lung fibrosis—the bleomycin-induced lung fibrosis model abundantly used by many labs and the JUN-induced lung fibrosis model—both demonstrated increased activation and phosphorylation of JUN after initiation of lung fibrosis in mice. (b) The time course of bleomycin induction in mice and in vivo treatment with blocking antibodies. (c, d) Morphological and molecular markers of representative histologic sections of wildtype and B6.129S2-Il6tm1Kopf/J (IL-6KO) mice lung tissues after fibrosis induction and treatment with blocking antibodies against immune checkpoint inhibitors and IL-6. Hematoxylin-Eosin (H.E.) stains and CD47, FSP1 counterstained with DAPI (c), Masson’s Trichrome stains and PD-L1 and FSP1 with DAPI (d) demonstrating improved fibrosis along with decreased CD47 and PD-L1 immune checkpoint protein expression in fibroblasts (FSP1+). Scale bar, 100 μm. (e) Quantitation of PD-L1 and CD47 expression in fibroblasts and collagen fibrosis of 10 high power fields (40x) of trichrome-stained sections. Data are expressed as mean ± SD, ordinary one-way ANOVA (Dunnett’s multiple comparisons test), n.s., non-significant; **** $P < 0.0001$. The immune stains were evaluated by a blinded pathologist, in addition to image J software. (f) In vivo analysis of human fibrotic fibroblasts in kidney capsule adoptive transfer assay in NSG mice to study efficacy of PD-1/PD-L1 blockade with HAC protein. Representative bioluminescence imaging (BLI) image and quantification of luminescence intensity, trichrome and anti-GFP staining of kidney area with the xenograft demonstrate that PD-1/PD-L1 blockade with HAC increased fibrotic fibroblast clearance compared to placebo (PBS). Data are expressed as mean ± SD and analyzed by using two-way ANOVA followed by Tukey’s multiple comparisons test. ** $P < 0.01$. Scale bar, 100 μm. See Supplementary Table 4 for statistical details.
Supplementary Figure S7

Representative contour plots showing gating strategy for all the populations involved in this paper.
Figure 7

Pro-Fibrotic environment
* FAF remains invisible for immune system.
* FAF promotes and spreads fibrotic areas.

Quiescent Macrophage
- SIRPα
- PD-1
- CD47
- Don't eat me signals

Anti-Fibrotic environment
* FAF are eliminated by immune system.
* Fibrotic areas are reduced

Phagocytic Macrophage
- MHC-TCR Complex loaded with FAF antigens
- Don't eat me signals
- Anti-CD47 mAb
- Anti-IL-6 mAb

Fibrotic Associated Fibroblast (FAF)
- Exhausted T cell
- Treg cell

Activated T cell